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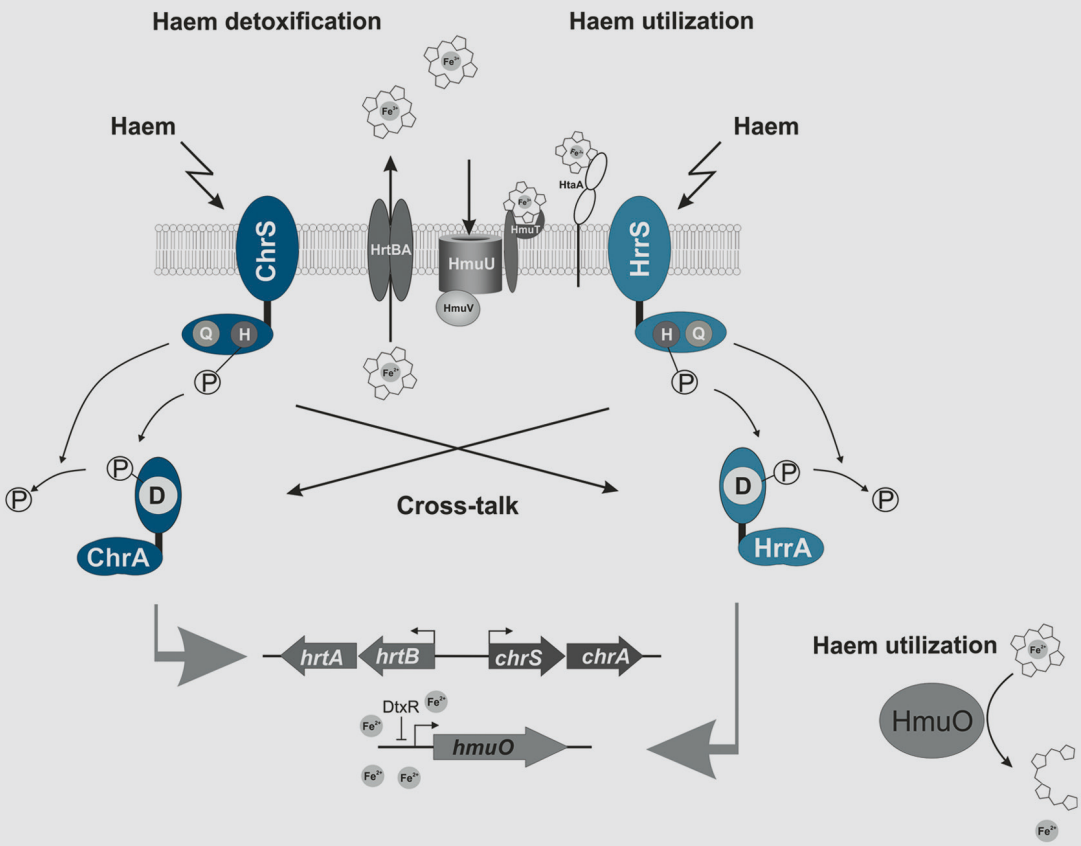
Eva Hentschel



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Content

Abbreviations

Amp^R	Ampicillin resistance
ATCC	American Type Culture Collection
BHI(S)	Brain Heart Infusion (+ Sorbitol)
CA	Catalytical domain
DNase	Desoxyribonuclease
DHp	Dimerization histidine phosphotransfer domain
EMSA	Electrophoretic mobility shift assay
et al.	*et alii*
eYFP	Enhanced yellow fluorescent protein
HK	Histidine kinase
IPTG	Isopropyl-thio-β-D-galactopyranosid
Kan^R	Kanamycin resistance
LB	Luria Bertani
MBP	Maltose binding protein
OCS	One-component system
OD_{600}	Optical density at 600 nm
OPD	Output domain
RBS	Ribosome binding site
REC	Receiver domain
RR	Response regulator
TCS	Two-component system
TE	Tris base - EDTA
TMD	Transmembrane domain
TNI	Tris base - NaCl - Imidazol
v/v	Volume per volume
WT	Wild type
w/v	Weight per volume

Further abbreviations not included in this section are according to international standards, as for example listed in the author guidelines of the *FEBS Journal*.

Author contributions

Destabilized eYFP variants for dynamic gene expression studies in *Corynebacterium glutamicum*

JF, AB and NR designed the study which was supervised by JF, NR and NM. The experimental work was performed by EH (construction and application of destabilized eYFP) and CW (construction and characterization of destabilized GFPuv). EH, CW and NR analyzed the data. JF and EH wrote the manuscript.

The two-component system ChrSA is crucial for haem tolerance and interferes with HrrSA in haem-dependent gene regulation in *Corynebacterium glutamicum*

AH and JF designed the study which was supervised by JF. The experimental work was performed by AH and CG. EH performed the promoter fusion analyses. JK provided RNASeq data. AH performed the analysis of the data. AH and JF wrote the manuscript, assisted by EH and MBo.

Phosphatase activity of the histidine kinases ensures pathway specificity of the ChrSA and HrrSA two-component systems in *Corynebacterium glutamicum*

EH, MB and JF designed the study which was supervised by JF. The experimental work was performed by EH, CG, and CM. EH performed the analysis of the data. EH and JF wrote the manuscript, assisted by MB and MBo.

Identification of residues involved in phosphatase activity of the two-component systems HrrSA and ChrSA in *Corynebacterium glutamicum*

EH and JF designed the study which was supervised by JF. The experimental work was performed by EH and DW. EH performed the analysis of the data. EH and JF wrote the manuscript.

AB: Andreas Burkovski, AH: Antonia Heyer, CG: Cornelia Gätgens, DW: Dominik Wolf, EH: Eva Hentschel JF: Julia Frunzke, JK: Jörn Kalinowski, MBo: Michael Bott, CM: Christina Mack, MB: Melanie Brocker, NR: Nadine Rehm, NM: Nurije Mustafi, CW: Cornelia Will.

1 Summary

1.1 Summary English

Two-component systems (TCS) are the prevalent mode for bacteria to sense and respond to changes in their natural habitat. An important protein-cofactor and alternative iron source, sensed by TCS, is haem. In the Gram-positive soil bacterium *Corynebacterium glutamicum* the TCS HrrSA is crucial for the utilization of haem. Besides HrrSA, a homologous haem-dependent TCS termed ChrSA could be identified. For the analysis of transient gene expression of ChrSA targets, appropriate reporters had to be constructed first.

Autofluorescent proteins are valuable tools for the *in vivo* monitoring of gene expression. However, due to the relatively long half live of most fluorescent proteins, visualization of transient changes remains difficult. SsrA-mediated peptide tagging was used for the construction of destabilized eYFP. The *C. glutamicum* SsrA tag variants (AAEKSQRDYAASV and -AAV) turned out to be suitable for monitoring dynamic gene expression in *C. glutamicum*. The respective eYFP variants displayed half-lives of ~22 min (ASV) and ~8 min (AAV).

Reporter studies using native eYFP provided strong evidence that ChrSA is the main activator of the divergently located operon *hrtBA*, encoding for a putative haem ABC transporter, which is required to counteract toxic intracellular accumulation of haem. Furthermore, ChrA acts as a repressor of the homologous response regulator *hrrA* providing first evidence for a close interplay of the TCS HrrSA and ChrSA.

The major focus of this work was to assess the close interplay of HrrSA and ChrSA in haem-dependent signal transduction and to uncover mechanisms enforcing specificity. ChrSA and HrrSA share a high sequence similarity and inherit distinct roles in the control of haem-homeostasis. Both TCS exhibit a high level of cross-talk, counteracted by the phosphatase activity of the sensor kinases HrrS and ChrS, which was shown to be specific for their cognate response regulators. Mutation of a conserved glutamine residue within the phosphatase motif (DxxxQ) of HrrS and ChrS led to a highly increased activation of target gene reporters, confirming the catalytical role of this glutamine residue for phosphatase activity.

As the phosphatase motif of HrrS and ChrS is completely identical, further catalytical residues involved in phosphatase reaction were identified. Besides phosphatase activity, pathway specificity can further be enhanced by molecular recognition. Analysis of chimeric proteins of HrrS and ChrS delivered first evidence, that residues forming the interface during phosphatase reaction are located inside the dimerization and histidine phosphotransfer (DHp) domain. Taken together, the results emphasize the importance of phosphatase activity and molecular recognition as crucial mechanisms to ensure pathway specificity of these haem-dependent and highly related TCS HrrSA and ChrSA in *C. glutamicum*.

1.2 Summary German

Zweikomponenten Systeme (ZKS) ermöglichen es Bakterien veränderte Umweltbedingungen wahrzunehmen und darauf zu reagieren. Häm, welches von ZKS detektiert wird, stellt einen wichtigen Protein-Cofaktor und alternative Eisenquelle dar. In dem Gram-positiven Bodenbakterium *Corynebacterium glutamicum* ist das ZKS HrrSA für die Verwertung von Häm verantwortlich. Zudem konnte neben HrrSA ein weiteres homologes Häm-abhängiges ZKS, ChrSA, identifiziert werden. Zur Analyse der transienten Genexpression der ChrSA Zielgene wurden zunächst geeignete Reporter konstruiert.

Autofluoreszenzproteine sind ein verlässliches Werkzeug um die Genexpression *in vivo* zu messen. Aufgrund der relativ langen Halbwertszeit von Fluoreszenzproteinen ist eine Visualisierung der transienten Genexpression schwierig. Mittels eines SsrA Peptid-Tags wurde destabilisiertes eYFP konstruiert. Die *C. glutamicum* SsrA Tag Varianten (AAEKSQRDYAASV und –AAV) erwiesen sich als geeignet für die Analyse der dynamischen Genexpression in *C. glutamicum*. Für die jeweiligen eYFP Varianten wurden Halbwertszeiten von ~22 min (ASV) und ~8 min (AAV) ermittelt.

Reporterstudien mit nativem eYFP lieferten wichtige Hinweise darauf, dass ChrA der Hauptaktivator des divergent lokalisierten Operons *hrtBA* ist, welches für einen putativen Häm- ABC Transporter kodiert und zur Vermeidung von toxischen intrazellulären Häm-Konzentrationen dient. Darüber hinaus reprimiert ChrA den homologen Antwortregulator *hrrA*, welches einen ersten Anhaltspunkt des engen Zusammenspiels der ZKS HrrSA und ChrSA lieferte.

Das Hauptaugenmerk dieser Arbeit lag auf der Analyse der Interaktion von HrrSA und ChrSA während der Häm-abhängigen Signaltransduktion und der Aufklärung spezifitäts-vermittelnder Mechanismen. ChrSA und HrrSA besitzen eine hohe Sequenzähnlichkeit und übernehmen distinkte Funktionen bei der Kontrolle der Häm-Homöostase. Beide ZKS zeigen ein hohes Level an Kreuz-Phosphorylierung, dem die Phosphatase-Aktivität der Sensorkinasen HrrS und ChrS entgegenwirkt. Diese ist sehr spezifisch für den eigenen Antwortregulator. Eine Mutation des konservierten Glutamin-Restes innerhalb des Phosphatase-Motives (DxxxQ) von HrrS und ChrS führte zu einer erhöhten Aktivierung des Zielgenreporters, was die katalytische Funktion dieses Glutamin-Restes für die Phosphatase-Aktivität bestätigt.

Da das Phosphatase-Motiv von HrrS und ChrS identisch ist, wurden weitere katalytische Reste, die für die Phosphatase-Aktivität verantwortlich sind, identifiziert. Neben der Phosphatase-Aktivität wird die Spezifität des Signalweges durch molekulare Erkennung vermittelt. Analysen von HrrS- und ChrS-Chimären lieferten erste Hinweise darauf, dass Aminosäurereste, welche während der Phosphatase-Reaktion die Verbindung zwischen Kinase und Antwortregulator bilden, in der Dimerisierungs- und Histidin-Phosphotransfer (DHp) Domäne lokalisiert sind. Zusammengefasst unterstreichen diese Ergebnisse die Bedeutung von Phosphatase-Aktivität und molekularer Erkennung für die Signaltransduktionsspezifität dieser Häm-abhängigen und nah verwandten ZKS HrrSA und ChrSA in *C. glutamicum*.

2 Introduction

2.1 Sensing environmental cues - Bacterial signal transduction

In a constantly changing environment like the soil, the sea, a host or the phyllosphere, bacterial survival critically relies on a sophisticated regulatory equipment, allowing a swift metabolic response and physiological adaptation. Bacterial genomes encode for a large repertoire of different kinds of signal transduction systems. The most prominent modes of sensing and responding towards environmental stimuli can be subdivided into one-component systems (OCS), extracytoplasmic function (ECF) σ factors, and two-component systems (TCS). These versatile signaling systems are responsible for the regulation of different processes, among them the response towards different stress stimuli, changes in osmolarity, nutrient availability and many more (Capra and Laub, 2012, Jordan *et al.*, 2008).

2.2 One-component systems

The most common devices connecting environmental stimuli to adaptive responses are OCS. OCS are single proteins including both, an input and an output domain. Most of the OCS harbour a helix-turn-helix DNA binding domain, which is usually located at the N- or C-terminal end of the protein. Prokaryotic OCS can be subdivided into at least 20 families. These regulatory systems detect stimuli (light, gas and small molecules) exclusively in the cytosol and appear more frequently in prokaryotes than TCS (Ulrich *et al.*, 2005). Prominent examples from two major OCS families are the lactose repressor LacI or TetR, which are involved in regulating antibiotic resistance in *E. coli* (Cuthbertson and Nodwell, 2013, Lewis *et al.*, 1996). It was suggested that OCS are evolutionary precursors of the more complex TCS (Ulrich *et al.*, 2005).

2.3 ECF σ factors

Bacterial σ factors are a crucial feature for ensuring promoter specificity of the RNA polymerase (Ishihama, 2010). Besides that, most species also possess alternative σ factors such as the large group of ECF σ factors, which represent a subfamily of σ^{70} factors. ECF σ factors are responsible for sensing and responding to changes in the

bacterial periplasm and the extracellular space (Brooks and Buchanan, 2008, Staron *et al.*, 2009, Mascher, 2013) and are often co-transcribed with their cognate anti-σ factors (mostly transmembrane proteins) that bind, and inhibit the σ factor in the absence of a specific stimulus (Helmann, 1999). Upon stimulus perception, the σ factor is released and can bind to the RNA polymerase to stimulate transcription. Thereby, the primary σ factor is replaced by the ECF σ factor and the RNA polymerase is redirected to alternative promoters (Helmann and Chamberlin, 1988). Well characterized ECF signaling pathways from *Escherichia coli* include for instance σ^E, mediating the response towards periplasmic stress and heat, or FecI which is involved in the regulation of iron transport (Braun *et al.*, 2003, Raivio and Silhavy, 2001).

2.4 Two-component systems

2.4.1 Architecture of two-component systems

One of the best characterized signal transduction modes are TCS, enabling bacteria to stay in touch with their environment and allow the perception and processing of a multitude of different intra- and extracellular stimuli (Stock *et al.*, 2000, Mascher *et al.*, 2006). Since their first discovery almost 30 years ago, the function of numerous TCS was disclosed. The first prokaryotic TCS and their function were identified during genetic screens of mutants of *E. coli* and *Bacillus subtilis* in the mid-eighties. Early studies for instance discovered the NtrC/NtrB TCS (prior named GlnG/GlnL) from *E. coli,* which was demonstrated to be responsible for the control of nitrogen assimilation (Ninfa and Magasanik, 1986, Keener and Kustu, 1988, Ferrari *et al.*, 1985, Nixon *et al.*, 1986). These discoveries have laid the foundation for today´s TCS research that continues vigorously up to now.

The prototypical TCS is composed of a membrane-bound histidine kinase (HK) responsible for signal detection and a cognate response regulator (RR) crucial for transducing this signal to the output level, which comprises the regulation of gene expression, catalytical activity or protein-protein interaction (Hoch and Silhavy, 1995, Inouye and Dutta, 2003).

Architecture of histidine kinases

In principle, HKs are composed of a transmembrane N-terminal sensor domain (TMD) and a cytoplasmic transmitter domain (Fig. 1). The transmitter domain comprises the dimerization and histidine phosphotransfer domain (DHp), also termed HisKA in Pfam database and the C-terminal catalytical ATP binding domain (CA), also defined as HATPase_c domain (Finn *et al.*, 2014, Punta *et al.*, 2012). Most kinases contain at least one additional domain between the TMD and DHp domain (PAS, HAMP, or GAF) (Galperin *et al.*, 2001). These domains can either be required for the transduction of signals from the TMD to the DHp and CA domains or directly recognize cytoplasmic signals (Parkinson, 2010, Möglich *et al.*, 2009). Both the DHp and the CA domains commonly contain several conserved boxes. The DHp domain contains the conserved H box, including the conserved histidine residue and the X box responsible for dimerization. The CA domain contains the conserved N, D, F and G boxes, which are involved in the ATP binding and HK autophosphorylation (Wolanin *et al.*, 2002, Parkinson and Kofoid, 1992).

Sequence analysis of these conserved boxes of the transmitter domain was used to perform an allocation of HKs into eleven subgroups (HPK1-11) (Grebe and Stock, 1999). In the last years this approach for the identification of HKs has mainly been replaced by an approach based on domain analysis. According to the Pfam database for instance, the HisKA (DHp) domain was divided into four domain families: HisKA (PF00512), HisKA_2 (PF07568), HisKA_3 (PF07730), and HWE_HK (PF007536) (Finn *et al.*, 2014). A further classification scheme, taking into consideration functional aspects is based on the sensor domain architecture of HK. This scheme allows a subgrouping of HKs in periplasmatic-, cytoplasmatic- and intramembrane sensing HKs (Mascher *et al.*, 2006).

Architecture of response regulators

A prototypical RR consists of two subdomains, the N-terminal receiver domain (REC), also termed response regulator domain in Pfam, and the C-terminal output domain (OPD) (Fig. 2.4.1). More than 60% of RRs are transcription factors with DNA-binding effector domains (Gao *et al.*, 2007). Based on DNA-binding domains, RRs can be subdivided into three major families, named after extensively characterized members:

OmpR, NarL, and NtrC (Galperin, 2006). The OmpR family is characterized by a winged-helix DNA-binding domain and the NarL subfamily by a four-helix helix-turn-helix domain (Martinez-Hackert and Stock, 1997, Milani *et al.*, 2005). The NtrC subfamily function as transcriptional enhancers and activate σ^{54} promoters by promoting an open complex formation in RNA polymerases. They are characterized by an ATPase domain fused to a factor of inversion (Fis)-type helix-turn-helix domain (Batchelor *et al.*, 2008). For NtrC it was shown that phosphorylation stimulates oligomerization leading to ATP hydrolysis, delivering energy for the open complex formation and initiation of transcription (Weiss *et al.*, 1991, Wedel and Kustu, 1995, Austin and Dixon, 1992, Wyman *et al.*, 1997).

However, a significant fraction of bacterial RRs do not regulate transcription directly. Some RRs include output domains that exhibit enzymatic activity: methylesterases, adenylate cyclases, c-di-GMP-specific phosphodiesterases, histidine kinases or serine/threonine protein kinases (Galperin, 2010).

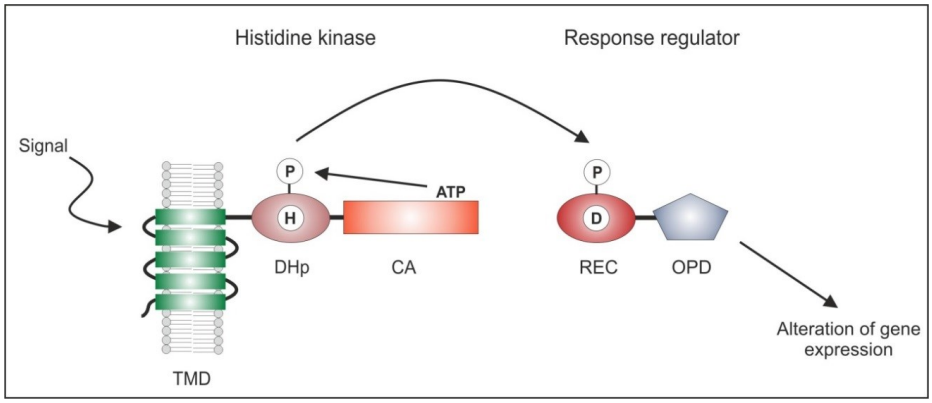

Figure 2.4.1: Two-component signal transduction. Upon signal perception of the histidine kinase, which typically occurs in the transmembrane domain (TMD), the catalytical domain (CA) mediates the autophosphorylation of a conserved histidine residue, located inside the dimerization and histidine phosphotransfer domain (DHp). The phosphate residue is the subsequently transferred to the conserved aspartate residue, located in the receiver domain (REC) of the response regulator. The output domain (OPD) of the response regulator binds to then promoter region of target genes, to regulate gene expression in response to the certain stimulus. Adapted from (Jensen *et al.*, 2002).

2.4.2 Signaling fidelity in two-component systems

Upon binding or reacting with a specific stimulus the CA-domain of the HK binds ATP and transfers the γ-phosphoryl group from ATP to the conserved histidine residue within the DHp domain (Fig. 1) (Stock *et al.*, 2000). This phosphoryl group is then subsequently transferred to the conserved aspartate residue located inside the REC domain of the RR. The phosphorylation of the RR, which is catalyzed by the RR itself, induces a conformational change and drives RR homodimerization resulting in an activation of the output domain. Consequential, the active state RR can function as transcriptional activator or repressor, depending on the promoter architecture and the RR binding site relative to the transcriptional start site (West and Stock, 2001, Mascher *et al.*, 2006).

A permutation of a TCS is the phosphorelay system, here a hybrid HK auto-phosphorylates and then transfers the phosphoryl group to an internal receiver domain. The phosphoryl group is then transferred to a histidine phosphotransferase and subsequently to the RR (Varughese, 2002). Another common signal transduction variant can also be a branched pathway, meaning the signal input of many different HKs converges at a single RR, or one single HK phosphorylates multiple RRs (Laub and Goulian, 2007). This is the case for the HK CheA, which phosphorylates the two RRs CheY and CheB in *E. coli* to provoke a chemotactic response (Baker *et al.*, 2006).

2.4.3 Evolution of two-component systems

TCS can be found in nearly all domains of life and were identified in eubacteria, archaea and a few eukaryotes as plants or yeasts (Loomis *et al.*, 1998, Koretke *et al.*, 2000). Phylogenetic analysis proposed that TCS originated in bacteria and were transferred to archaea and eukaryotes *via* lateral gene transfer. This observation is also supported by the greater distribution of TCS in bacteria (Koretke *et al.*, 2000, Ulrich *et al.*, 2005).

As more and more sequenced bacterial genomes become available, the number of identified TCS increases steadily. Prokaryotic organisms often encode dozens and sometimes up to hundreds of these TCS (Galperin, 2005, Capra and Laub, 2012). In fact, TCS screening studies revealed a positive correlation between the number of TCS harboured by a bacterial genome and the genome size, as well as the complexity of

their ecological niche (Galperin, 2005, Alm *et al.*, 2006). Bacteria living in diverse and fluctuating environments typically encode a large repertoire of TCS as for instance *Myxococcus xanthus* (136 HKs and 127 RRs). In contrast, no TCS are found in the genome of *Mycoplasma* species (Ulrich and Zhulin, 2010).

New TCS typically arise from gene duplication events or *via* lateral gene transfer (Alm *et al.*, 2006). Lateral gene transfer can occur by various mechanisms including conjugation, competence, or phage infection (Arthur *et al.*, 1992, Deiwick *et al.*, 1999, Salanoubat *et al.*, 2002, Wright *et al.*, 1993). Newly acquired TCS can either be removed from the genome (mutation and deletion) or fixed within the genetic repertoire, providing its host with novel sensing functions. Especially after gene duplication events, insulation of new TCS is crucial to avoid detrimental cross-talk between different signal transduction pathways (Conant and Wolfe, 2008, Hooper and Berg, 2003). This can for instance be achieved by i) changes in the HK sensory domain, ii) changes in RR pathway outputs or iii) mutations in interface residues, to ensure pathway specificity of new and ancestral TCS (Capra and Laub, 2012). The present work will mainly focus on the aspect of insulation *via* interface residues to ensure specificity in TCS signal transduction.

2.4.4 Specificity of two-component signal transduction

As a large number of TCS originate from gene duplication events and lateral gene transfer, HKs and RRs often share a high sequence similarity (Alm *et al.*, 2006). Thus, cross-talk between non-cognate partners is unavoidable especially immediately after TCS duplication events (Capra *et al.*, 2012, Galperin, 2005). As this cross-talk can attenuate the response of the original signal, insulation of new TCS is the key to specific and efficient signal transduction (Capra and Laub, 2012).

A prominent mechanism to ensure insulation of signal transduction pathways is molecular recognition (Fig. 2.4.4A). This is based on the assumption that a HK has the intrinsic ability to discriminate its cognate RR from the crowded milieu of non-cognate RRs. A relatively small subset of residues at the interface between HKs and RRs is responsible for maintaining this specificity. These residues, forming a region of sequence space that insulates them from other TCS, were shown to be located in the first helix of the HK and the cognate RR (Skerker *et al.*, 2008, Laub and Goulian, 2007,

Capra *et al.*, 2012). During the course of evolution any mutation in the interface of a cognate HK-RR pair has to be compensated by a further mutation of the corresponding partner. This principle of maintaining specificity during divergence of new TCS was described as coevolution (Capra and Laub, 2012, Podgornaia and Laub, 2013).

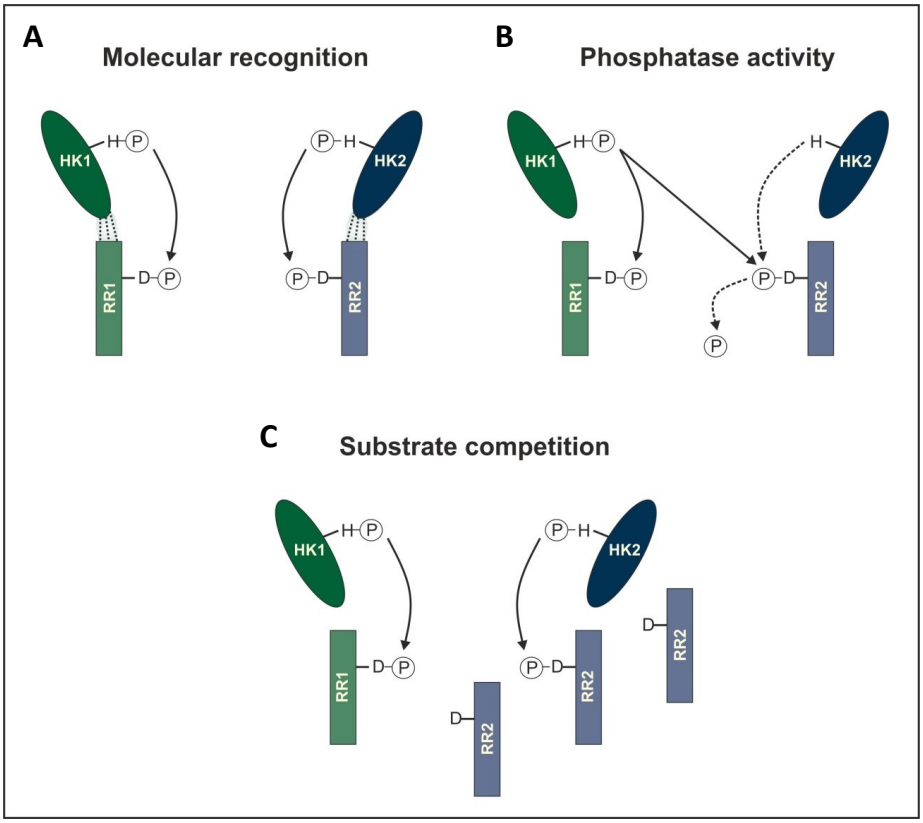

Figure 2.4.4: Mechanisms ensuring pathway specificity in two-component signal transduction. A: Molecular recognition is the intrinsic ability of a histidine kinase (HK) to discriminate the cognate response regulator (RR) *via* so called interface residues. B: Phosphatase activity describes the bifunctional nature of some HKs. In the absence of a stimulus the HK dephosphorylates the cognate RR to prevent cross-talk from other kinases or small phosphor-donors. C: Substrate competition between RRs for a particular HK can further ensure specificity. The cognate RR outcompetes non-cognate RRs for phosphorylation. Adapted from (Podgornaia and Laub, 2013).

Further mechanisms ensuring phosphotransfer specificity in TCS signal transduction are phosphatase activity of the HK and substrate competition (Fig. 2.4.4B). Phosphatase activity describes the bifunctional nature of HKs, which often catalyse not only phosphorylation, but also dephosphorylation of their cognate RR.

This ensures the elimination of an inappropriate RR phosphorylation by non-cognate HKs or small phospho-donors like acetyl phosphate (Huynh and Stewart, 2011, Igo *et al.*, 1989). The HK catalyzes dephosphorylation of the RR through an in-line attack by a nucleophilic water molecule (Wolanin *et al.*, 2003).

Substrate competition depends on the stoichiometry of HK and RR (Fig. 2.4.4C). In most cases, the RR outvalues the level of HK and thereby outcompetes non-cognate partners to prevent cross-talk (Groban *et al.*, 2009, Siryaporn and Goulian, 2008).

2.5 Stimuli of two-component systems

2.5.1 Iron - a critical trace element

Environmental stimuli are typically sensed by the N-terminal TMD domain of the HK. This can occur in the periplasmatic-, cytoplasmatic-, or transmembrane region of the HK (Mascher *et al.*, 2006). The stimuli sensed by TCS are diverse, among them different stress stimuli, osmolarity changes, antibiotics, the cellular redox state, quorum signals, and many more. One important feature is the ability of a TCS to sense the availability of nutrients, including trace elements as magnesium, calcium, copper, zinc or iron (Calva and Oropeza, 2006, Steele *et al.*, 2012).

The trace element iron is essential for almost all living species. Although iron is one of the most abundant elements in the planets crust, acquisition and incorporation is challenging for bacteria based on the toxicity of ferrous iron (Fe^{2+}) and the insolubility of ferric iron (Fe^{3+}) (Chipperfield and Ratledge, 2000, Pierre and Fontecave, 1999). Iron is involved in numerous cellular reactions, as it forms a catalytic center for redox reactions in many enzymes involved in electron transport, TCA-cycle, peroxide reduction, and nucleotide biosynthesis (Cornelis *et al.*, 2011).

2.5.2 Haem - an alternative source of iron

A rich source for iron is haem (Fe^{2+} bound in protoporphyrin IX) (Fig. 2.5.2), especially for pathogens sequestering haem from their hosts (Nobles and Maresso, 2011). Mammalian serum concentrations of free iron and haem are for instance below 10^{-18} M as they are sequestered in many proteins as haemoglobin (~80%), myoglobin, transferrin, ferritin, albumin, and haptoglobin (Tong and Guo, 2009). Also for non-

pathogenic bacteria endemic in the soil, where haem is present in decaying organic material, it represents an attractive alternative iron source (Andrews *et al.*, 2003). The form of haem transported by bacterial uptake systems is hemin, the oxidized form of haem, which is present in extracellular environments. Haem is also an important cofactor of proteins of the respiratory chain, catalases or peroxidases (Rouault, 2004). Two types of haem serve as cofactors in enzymes: Haem a and c, which are derived from side-chain modifications of the most abundant form haem b. Haem is a lipophilic molecule with low molecular weight (616.48 Da) and it was shown that it can passively diffuse across model lipid bilayers (Ryter and Tyrrell, 2000).

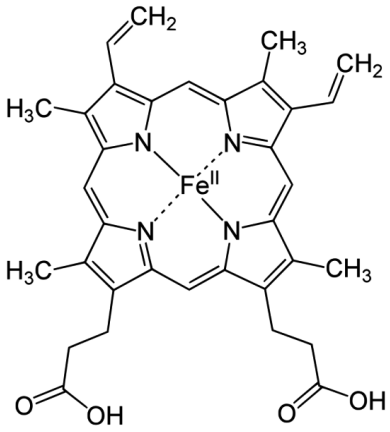

Figure 2.5.2: Structure of haem *b* (protohaem IX). Iron (Fe^{2+}) is complexed by four nitrogen atoms of the porphyrin ring structure.

Bacteria have evolved sophisticated strategies for the uptake of haem, which is different between Gram-positive and Gram-negative species. Gram-negative bacteria sequester haem mainly *via* direct binding of haem by specific receptors located on the outer membrane and ABC transporters for the transport into the cytoplasm as described for PhuR from *Pseudomonas aeruginosa* (Smith and Wilks, 2015). Another common mode of haem acquisition is comprised of a mostly Ton-B dependent outer membrane receptor, an ABC transporter and extracellular hemophores as described for HasA from *Serratia marcescens* (Letoffe *et al.*, 1994a, Letoffe *et al.*, 1994b). As Gram-positive bacteria lacking the outer membrane but contain a peptidoglycan layer, the acquisition of haem is different. A prominent haem acquisition system is the HmuTUV/HtaAB system described for *Corynebacterium diphtheriae* and *ulcerans*. HtaA

and HtaB are surface located and function as hemin receptors and HmuTUV functions as an ABC transporter (Allen and Schmitt, 2011, Allen and Schmitt, 2009, Schmitt and Drazek, 2001). Another intensively characterized mechanism for haem-uptake, described for some Gram-positive bacteria is termed iron-responsive determinants (Isd) uptake system. *Staphylococcus aureus* for instance binds hemin or haemoglobin to its cell envelope through four Isd proteins, which are covalently linked to the peptidoglycan. Haem is transported through the cell envelope *via* cascade transfer between various Isd receptors (Mazmanian *et al.*, 2003, Muryoi *et al.*, 2008).

Once entered the cytoplasm, haem is incorporated into haem containing proteins or enzymes, sequestered by cytoplasmatic haem-chaperons or degraded. Haem degradation is commonly catalyzed by the haem-oxygenase, which also uses haem as cofactor for this reaction. Haem is degraded to biliverdin, Fe^{2+}, and carbon monoxide in equimolar amounts. The reaction requires a total of three oxygen molecules and seven electrons for the cleavage of one haem molecule (Wilks and Heinzl, 2014). A further haem degradation protein using a distinct mechanism from classical haem oxygenases is termed IsdG and was identified, for instance, in *S. aureus* (Skaar and Schneewind, 2004).

Besides acquiring haem from their environments, bacteria are also able to synthesize haem *de novo*. The common precursor for tetrapyrrols, including haem, is 5-aminolevulinic acid, as it serves as a source of carbon and nitrogen for haem formation (Layer *et al.*, 2010). In the Gram-positive soil bacterium *C. glutamicum*, 5-aminolevulinic acid is synthesized *via* the C5 pathway from glutamate by glutamyl-tRNA synthetase (GltX), glutamyl-tRNA reductase (HemA), and glutamate-1-semialdehyde aminomutase (HemL). Protohaem IX (haem b) is converted into haem a *via* protohaem IX farnesyl transferase (CtaB) and haem o monooxygenase (CtaA) (Niebisch and Bott, 2001, Bott and Niebisch, 2003, Brown *et al.*, 2002).

2.6 Haem and iron homeostasis in *C. glutamicum*

The absolute need for haem and iron is accompanied by their toxicity when present at higher levels, as they are involved in the formation of reactive oxygen species and their accumulation can lead to cellular damage and oxidative stress (Andrews *et al.*, 2003, Pierre and Fontecave, 1999, Ryter and Tyrrell, 2000). Thus, haem and iron homeostasis

have to underlie a stringent regulation. In *C. glutamicum*, which is an important platform organism for biotechnology the transcriptional regulator DtxR is the master regulator of iron homeostasis. In the presence of iron, DtxR in complex with Fe^{2+} represses genes involved in iron acquisition as well as *ripA* encoding for RipA the repressor of iron-containing proteins, and activates genes encoding iron storage proteins (ferritin and Dps) (Brune *et al.*, 2006, Wennerhold and Bott, 2006, Wennerhold *et al.*, 2005).

Among the targets repressed by DtxR, the haem importer *hmuTUV* and the response regulator *hrrA* belonging to the haem-dependent TCS HrrSA was found (Wennerhold and Bott, 2006). It was shown that HrrSA is important for the utilization of haem as an alternative source of iron as it activates the expression of *hmuO* encoding the haem-oxygenase in the presence of haem. Furthermore, HrrSA activates genes encoding for respiratory chain components (*ctaD* and the *ctaE-qcrCAB* operon) and acts as a repressor of genes coding for haem biosynthesis enzymes (*hemA*, *haem*, and *hemH*) (Frunzke *et al.*, 2011). To allow the preferential utilization of iron, *hrrA* and *hmuO* expression both underlie the repression of DtxR (Wennerhold and Bott, 2006). Besides HrrSA, a second homologous TCS named ChrSA could be identified. Interestingly, transcriptome analysis of *hrrA* deletion mutants provided hints for a repression of *chrSA* by HrrA and thus revealed the first evidence for a close interplay of the HrrSA and ChrSA systems in *C. glutamicum* (Frunzke *et al.*, 2011). In preliminary studies, first evidence for a function of ChrSA in heme detoxification was provided during growth experiments with deletions mutants and studies aiming at the identification of target genes.

Besides the TCS HrrSA and ChrSA, in the genome of *C. glutamicum* 11 further TCS can be found (Bott and Brocker, 2012). Up to date, the role of only a few TCS was uncovered, among them the role of CitAB controlling citrate utilization and the MtrBA system controlling osmoregulation and cell wall metabolism (Brocker *et al.*, 2009, Brocker *et al.*, 2011). Further systems, which were characterized more in detail are the TCS PhoSR, responsible for coping with phosphtate starvation and the TCS CopSR, which is crucial for responding towards copper stress (Schaaf and Bott, 2007, Schelder *et al.*, 2011).

2.7 Haem dependent two-component systems

A tight control of haem-homeostasis is crucial for almost all living species not only to ensure sufficient supply of this alternative iron source but also to avoid high intracellular levels. The control of haem-homeostasis is typically mediated by TCS, which was shown in comprehensive studies for several species.

One of the most prominent and best characterized examples are the two haem-dependent TCS HrrSA and ChrSA from *C. diphtheria*, which is a human pathogen and uses haem and haemoglobin as essential iron sources. The two-component system ChrSA activates the expression of genes encoding for the haem-oxygenase (*hmuO*), which mediates the utilization of haem and activates the expression of a haem regulated transporter efflux pump (*hrtBA*), to prevent toxic intracellular haem levels. Besides that, the TCS HrrSA also contributes approximately 20% to the activation of the expression of *hmuO* (Bibb *et al.*, 2007, Bibb and Schmitt, 2010). Moreover, both TCS are involved in the haem-dependent repression of *hemA* encoding for a haem biosynthesis enzyme (Bibb *et al.*, 2005).

Many orthologs of the TCS ChrSA and the haem-exporter HrtBA can be found in Gram-positive bacteria. *Staphylocuccus aureus*, *Lactococcus lactis*, *Staphylococcus epidermidis*, and *Bacillus anthracis* also employ the ChrSA ortholog HssRS for the activation of the expression of the haem-exporter *hrtBA*, protecting these bacteria from the toxic effects of high levels of haem. In *S. aureus* this does not only lead to a reduced haem toxicity but also to a tempered staphylococcal virulence (Stauff *et al.*, 2007, Stauff and Skaar, 2009b).

2.8 Aims of this work

The global aim of the present work will be to uncover the level of interaction of the TCS HrrSA and ChrSA in *C. glutamicum* and to shed light on the mechanisms ensuring pathway specificity in their signal transduction processes. Therefore, in the first place the role of ChrSA in the detoxification of haem will be elucidated by investigating the influence of ChrSA on the expression of *hrtBA* encoding for a putative haem exporter and by creating activity profiles for both TCS HrrSA and ChrSA. Furthermore, the interaction of both systems at the transcriptional level will be investigated. Therefore, target gene reporters of both TCS (P_{hmuO}-*eyfp* for HrrSA and P_{hrtBA}-*eyfp* for ChrSA) will

be constructed and used for reporter assays with several deletion mutants of both TCS. To overcome the problem of the relatively long half-life of fluorescent proteins, in a side project destabilized fluorescent proteins (eYFP) will be constructed *via* SsrA-mediated peptide tagging and tested regarding their application in *C. glutamicum*. For the investigation of the interaction of HrrSA and ChrSA *in vivo* and *in vitro*, growth experiments and reporter assays with strains lacking the kinases HrrS or ChrS, as well as *in vitro* phosphorylation assays will be performed. To test whether phosphatase activity is a crucial mechanism to ensure pathway specificity of HrrSA and ChrSA, sequences of HrrS and ChrS will be analyzed regarding a putative phosphatase motif. Phosphatase activity will be further tested in *in vitro* phosphorylation assays and reporter studies with strains lacking phosphatase domains. Another aim of this work was the identification of further catalytical phosphatase residues and of residues conferring specificity during phosphatase reaction. Therefore, construction of an error prone library of the HrrS DHp domain and a screening for kinase ON/phosphatase OFF mutants is necessary. Besides that, chimeric proteins will be constructed to localize residues conferring specificity during phosphatase reaction. The investigation of signal transduction of the TCS HrrSA and ChrSA will provide a comprehensive understanding of their interplay and pathway specificity in *C. glutamicum*.

3 Results

The major topic of this PhD thesis was the analysis of the interaction and signal transduction specificity of the two-component systems HrrSA and ChrSA. One methodological-oriented study focused on the enlargement of the tool box of appropriate autofluorescent reporter proteins for the analysis of transient gene expression changes in *C. glutamicum*. The results allocated in this research have been summarized in three publications and a manuscript (to be submitted).

The publication “Destabilized eYFP variants for dynamic gene expression studies in *Corynebacterium glutamicum*” describes the construction of unstable eYFP variants via SsrA-mediated peptide tagging. Half-lives of destabilized eYFP variants were determined using western-blotting analysis and fluorescence measurements. The application was tested for a sensor enabling the investigation of gluconate catabolism (P_{gntK}-*eyfp*-ASV). This was a joint project with Andreas Burkovski, Nadine Rehm, and Cornelia Will, from the FAU Erlangen-Nürnberg, who contributed to this project with the construction and characterization of unstable GFPuv variants.

The publication “The two-component system ChrSA is crucial for haem tolerance and interferes with HrrSA in haem-dependent gene regulation in *Corynebacterium glutamicum*” reports the key role of ChrSA for the detoxification of haem. ChrA activates the expression of a putative haem exporter *hrtBA* to prevent toxic intracellular levels of haem and contributes to the activation of *hmuO* encoding for the haem-oxygenase. Furthermore, reporter studies and transcriptome analysis suggested that ChrA acts as repressor of the paralogous response regulator *hrrA*, delivering first hints for a close interplay of the two-component systems HrrSA and ChrSA.

The investigation of the interaction and pathway specificity of the two-component systems HrrSA and ChrSA is described in the publication “Phosphatase activity of the histidine kinases ensures pathway specificity of the ChrSA and HrrSA two-component systems in *Corynebacterium glutamicum*”. Distinct roles of HrrSA (utilization of haem) and ChrSA (detoxification of haem) in the control of haem-homeostasis could be confirmed via activity profiling experiments. An interaction between HrrSA and ChrSA

on the level of phosphorylation could be observed in kinase deletion mutants during reporter studies and growth experiments and in *in vitro* phosphorylation assays. Phosphatase activity of the kinases HrrS and ChrS was disclosed as the key mechanism for ensuring pathway specificity in these TCS. A conserved glutamine residue, located within a putative phosphatase motif (DxxxQ), was shown to be responsible for catalytical phosphatase activity.

Although phosphatase activity seems to be a highly specific mechanism only for the cognate response regulator, catalytical phosphatase residues identified up to now are completely identical. In the manuscript “Identification of residues involved in phosphatase activity of the two-component systems HrrSA and ChrSA in *Corynebacterium glutamicum*” further catalytical residues were identified using a FACS screening approach of a HrrS error prone library. Analysis of chimeric proteins emphasized the location of interface residues, which confer specificity during phosphatase reaction, within the dimerization and histidine phosphotransfer domains of HrrS and ChrS.

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Brief report

Destabilized eYFP variants for dynamic gene expression studies in *Corynebacterium glutamicum*

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Summary

Fluorescent reporter proteins are widely used for the non-invasive monitoring of gene expression patterns, but dynamic measurements are hampered by the extremely high stability of GFP and homologue proteins. In this study, we used SsrA-mediated peptide tagging for the construction of unstable variants of the GFP derivative eYFP (enhanced yellow fluorescent protein) and applied those for transient gene expression analysis in the industrial platform organism *Corynebacterium glutamicum*.

The Gram-positive soil bacterium *Corynebacterium glutamicum* was isolated in 1957 in Japan due to its ability to excrete large amounts of the amino acid L-glutamate (Kinoshita, 1957). Within the last decades *C. glutamicum* was proven to be an excellent production platform not only for amino acids, but also for a variety of other metabolites, including organic acids, vitamins and polymer precursors (Leuchtenberger, 1996; Eggeling and Bott, 2005; Burkovski, 2008). Efficient metabolic engineering, however, depends on a detailed understanding of gene expression patterns and adaptive responses of the respective organism. Reporter proteins, such as β-galactosidase (*lacZ*), bacterial luciferase (*luxCDABE*), and autofluorescent proteins (*gfp*, *yfp*, etc.) represent convenient tools for the detection and quantification of molecular and genetic events (Ghim *et al.*, 2010). Among these, fluorescent proteins offer the advantage of broad-host applicability, no need for substrate addition, and a non-destructive measurement at the single cell level (Chalfie *et al.*, 1994; Tsien, 1998). A major drawback of several reporter proteins is, however, their extremely long half-life (GFP > 24 h), which leads to accumulation of the protein within the cell and hampers the study of transient changes in gene expression. To address this issue, Andersen and co-workers used C-terminal SsrA peptide tagging for destabilization of GFP in *Escherichia coli* and *Pseudomonas putida* (Andersen *et al.*, 1998). This approach was in the following successfully applied to generate unstable GFP variants in, e.g. *Mycobacterium* species or for destabilization of the *Photorhabdus luminescens* luciferase (Triccas *et al.*, 2002; Blokpoel *et al.*, 2003; Allen *et al.*, 2007).

The SsrA tag is encoded by the tmRNA (tmRNA, *ssrA* or 10Sa RNA), which functions as both transfer and messenger RNA and acts as a rescue system of ribosomes stalled on broken or damaged mRNA (Keiler, 2008). By translation of the messenger part of tmRNA a peptide tag of 11 amino acids is added to the C-terminus of the premature protein (*E. coli* SsrA tag: AANDENYALAA), thereby rendering it susceptible for tail-specific proteases, such as ClpXP, ClpAP or FtsH (Gottesman *et al.*, 1998; Herman *et al.*, 1998). Previous studies showed that variation of the terminal three amino acids of the tag can be used to generate variants of different protein stability (Andersen *et al.*, 1998).

The *ssrA* gene is highly conserved in bacterial genomes and a homologue was also annotated in the *C. glutamicum* genome (Kalinowski *et al.*, 2003). In this study we used SsrA peptide tagging to construct a set of destabilized fluorescent protein variants (eYFP and GFPuv) with significantly reduced half-lives, compared with the native proteins. These proteins represent valuable tools for the monitoring of dynamic gene expression patterns in the biotechnological organism *C. glutamicum*.

For construction of unstable fluorescent protein variants the native *E. coli* (AANDENYALAA) and *C. glutamicum* SsrA tag (AAEKSQRDYALAA) or tags varying in their terminal three amino acids of the corynebacterial tag (in the

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Table 1. Plasmids and oligonucleotides used in this study.

Plasmid	Properties	Reference
pEKEx2	Kan[R]; expression vector with *lacI*[q], P_{tac} and pUC18 multiple cloning site	Eikmanns *et al.* (1991)
pEKEx2-*eyfp*	Kan[R] pEKEx2 containing *eyfp* with artificial RBS, under control of P_{tac}	This study
pEKEx2-*eyfp*-asv	Kan[R], pEKEx2 containing *eyfp*, with artificial RBS, under control of P_{tac}, modified to include the gene sequence for the C-terminal *C. glutamicum* SsrA tag variation AAEKSQRDYAASV	This study
pEKEx2-*eyfp*-aav	Similar to pEKEx2-*eyfp*-ASV using the alternative tag AAEKSQRDYAAAV	This study
pEKEx2-*eyfp*-lva	Similar to pEKEx2-*eyfp*-ASV using the alternative tag AAEKSQRDYALVA	This study
pEKEx2-*eyfp*-laa	Similar to pEKEx2-*eyfp*-ASV using the native *C. glutamicum* tag AAEKSQRDYALAA	This study
pEKEx2-*eyfp*-Ec_laa	Similar to pEKEx2-*eyfp*-ASV using the native *E. coli* tag AANDENYALAA	This study
pJC1	Kan[R], Amp[R]; *C. glutamicum* shuttle vector	Cremer *et al.* (1991)
pJC1-P_{gntK}-*eyfp*	Kan[R], pJC1 containing *eyfp* under control of the promoter of *gntK* (153 bp)	This study
pJC1-P_{gntK}-*eyfp*-asv	Similar to pJC1-P_{gntK}-*eyfp* using *eyfp* modified to include the gene sequence for the C-terminal *C. glutamicum* SsrA tag AAEKSQRDYAASV	This study
pEKEx2-*eyfp-tetR*	Kan[R], pEKEx2 derivative containing *yfp-tetR*, encoding a eYFP–TetR fusion protein under the control of the P_{tac}	Frunzke *et al.* (2008a)
pEKEx2-*gfp*	Kan[R] pEKEx2 containing *gfpuv* with artificial RBS, under control of P_{tac}	This study
pEKEx2-*gfp*-asv	Kan[R], pEKEx2 containing *gfpuv*, with artificial RBS, under control of P_{tac}, modified to include the gene sequence for the C-terminal *C. glutamicum* SsrA tag variation AAEKSQRDYAASV	This study
pEKEx2-*gfp*-aav	Similar to pEKEx2-*gfpuv*-ASV using the alternative tag AAEKSQRDYAAAV	This study
pEPR1-*gfp*	*gfpuv*, Kan[R], *rep*, *per*, T1 (T-*trpE*) (T-*trpA*), T2 (T-*rrnB*) (T-*leuB*); promoter probe vector	Knoppova *et al.* (2007)

Oligonucleotide	Sequence 5′ -> 3′[a]	Application[b]
eYFP-ASV-*C.g.*-EcoRI-rv	CGCGAATTCTTA*AACTGATGCAGCGTAATCACGTTGGCTCTTTTCTGCTGCTCTAGACTTGTACAGCTCGTC*	Rv for *eyfp-Cg*-ASV
eYFP-AAV-*C.g.*-EcoRI-rv	CGCGAATTCTTA*AACAGCTGCTGCGTAATCACGTTGGCTCTTTTCTGCTGCTCTAGACTTGTACAGCTCGTC*	Rv for *eyfp-Cg*-AAV
eYFP-LVA-*C.g.*-EcoRI-rv	CGCGAATTCTTA*AGCTACTAAAGCGTAATCACGTTGGCTCTTTTCTGCTGCTCTAGACTTGTACAGCTCGTC*	Rv for *eyfp-Cg*-LVA
eYFP-LAA-*C.g.*-EcoRI-rv	CGCGAATTCTTA*AGCTGCTAAAGCGTAATCACGTTGGCTCTTTTCTGCTGCTCTAGACTTGTACAGCTCGTC*	Rv for *eyfp-Cg*-LAA
eYFP-LAA-*E.c.*-EcoRI-rv	CGCGAATTCTTA*AGCTGCTAAAGCGTAGTTTTCGTCGTTTGCTGCTCTAGACTTGTACAGCTCGTC*	Rv for *eyfp-Ec*-LAA
eYFP-EcoRI-rv	CGCGAATTCTTATCTAGACTTGTACAGCTCGTC (EcoRI)	Rv for *eyfp*
eYFP-RBS-BamHI-fw	CGCGGATCC**AAGGAGATATGAT**ATGGTGAGCAAGGGCGAGGAG (BamHI)	Fw for *eyfp* and destabilized variants
eYFP-ASV-*C.g.*-SalI-rv	CGCGTCGACTTA*AACTGATGCAGCGTAATCACG* (SalI)	Rv for *eyfp-Cg*-ASV
eYFP-AAV-*C.g.*-SalI-rv	CGCGTCGACTTA*AACAGCTGCTGCGTAATCACG* (SalI)	Rv for *eyfp-Cg*-AAV
eYFP-NdeI-fw	CGCCATATGGTGAGCAAGGGCGAGGAG (NdeI)	Fw for *eyfp* and destabilized variants
P_{gntK}-BamHI-fw	CGCGGATCCACATACAGTCCCCGTGATGTGAC (BamHI)	Fw for promoter region of *gntK*
P_{gntK}-NdeI-rv	CGC CATATG GTCTTATCCTTTCTTTGGTGGCG (NdeI)	Rv for promotor region of *gntK*
GFPuv-AAV-*C.g.*-EcoRI-rv	GCGCGATATCGAATTCTCATTA*AACAGCTGCTGCGTAATCACGTTGGCTCTTTTCTGCTGCTTTGTAGAGCT* CATCCATGCCATG (EcoRI)	Rv for *gfpuv-Cg*-AAV
GFPuv-ASV-*C.g.*-EcoRI-rv	GCGCGATATCGAATTCTCATTA*AACTGATGCAGCGTAATCACGTTGGCTCTTTTCTGCTGCTTTGTAGAGCTC* ATCCATGCCATG (EcoRI)	Rv for *gfpuv-Cg*-ASV
GFPuv-Acc65I-fw	GCGCGGTACCGGTAGAAAAATGAG (Acc65I)	Fw for *gfpuv* and destabilized variants
GFPuv-EcoRI-rv	GCGCGCGAATTCGAGAGTTATCTCGGCCAGCCAC (EcoRI)	Rv for *gfpuv*

a. Some oligonucleotides were designed with restriction sites (underlined), ribosome binding sites (bold) and include SsrA-tag sequences (italic) as indicated.
b. Rv, reverse primer; Fw, forward primer for amplification.

Table 2. Overview of SsrA tag variants.

SsrA-tag	Amino acid sequence	Half-life (min)[a]	Signal intensity (%)[b]
E. c. LAA-tag native	AANDENYA**LAA**	n.d.	n.d.
C. g. LAA-tag native	AAEKSQRDYA**LAA**	n.d.	n.d.
C. g. LVA-tag variation	AAEKSQRDYA**LVA**	n.d.	n.d.
C. g. ASV-tag variation	AAEKSQRDYA**ASV**	22 ± 4	46
C. g. AAV-tag variation	AAEKSQRDYA**AAV**	8 ± 3	20

a. Protein half-lives were calculated from Western blot analysis (Fig. 1C) corresponding to the decay law $\lambda = \frac{\ln\left(\frac{N_{(0)}}{N_{(t)}}\right)}{t}$ $T_{\gamma 2} = \frac{\ln(2)}{\lambda}$. Given values represent the average values with standard deviation of three independent experiments. For indicated constructs rapid degradation occurred and half-lives could not be determined (n.d.).
b. Signal intensity relative to native eYFP at the time of measurement.

following designated as ASV, AAV and LVA) were fused to the C-terminus of eYFP and GFPuv, which are both commonly used as reporter proteins in *C. glutamicum*. The fusion variants were cloned into the vector pEKEx2, under control of P_{tac}, and transferred into *C. glutamicum* ATCC 13032 by electroporation (Table 1). For fluorescence measurements cells were grown in 48-well microtiter Flowerplates containing CGXII minimal medium with 4% glucose in a BioLector microbioreactor system (m2p labs, Germany) (Kensy *et al.*, 2009); gene expression was induced by addition of 1 mM IPTG. In case of the eYFP variants we observed significant fluorescent signals for fusion constructs with ASV tag (46% of native eYFP) and AAV tag (20% of native eYFP) (Table 2). No signal was detected for the eYFP variants fused either to the native SsrA tag (*E. coli* or *C. glutamicum*) or to the LVA modified version (data not shown). To assess the stability of the ASV and AAV variants, transcription and translation of the

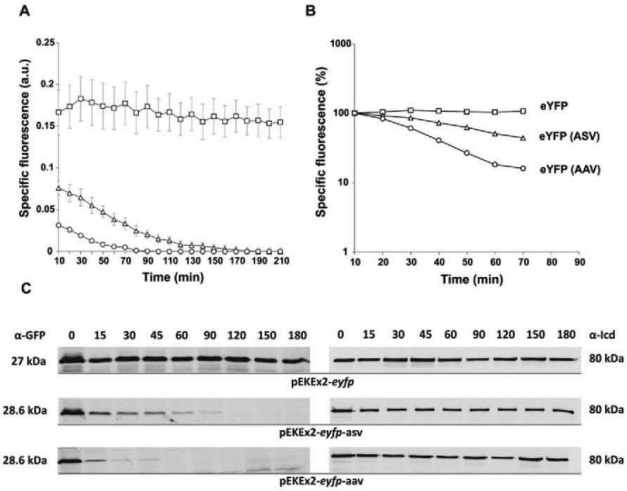

Fig. 1. Stability of eYFP variants in *C. glutamicum*. (A) Fluorescence of recombinant *C. glutamicum* ATCC 13032 strains expressing *eyfp* variants: ATCC 13032/pEKEx2-*eyfp* (squares), ATCC 13032/pEKEx2-*eyfp*-asv (triangles), ATCC 13032/pEKEx2-*eyfp*-aav (circles). Prior induction, cells were inoculated to an OD_{600} of 1 in 750 µl of CGXII minimal medium containing 4% glucose and cultivated in 48-well microtiter plates in the BioLector system (m2p labs, Germany). Gene expression was induced by addition of 1 mM IPTG. To estimate the stability of the eYFP variants 250 µg ml^{-1} rifampicin (Rif) and 100 µg ml^{-1} tetracycline (Tet) were added 1.5 h after induction to stop the transcription and translation. In the BioLector system the growth (backscatter signal of 620 nm light) and eYFP fluorescence (excitation 510 nm/emission 532 nm) were monitored in 10 min intervals. The specific fluorescence was calculated as fluorescence signal per backscatter signal (given in arbitrary units, a.u.). Results represent average values with standard deviation of three independent experiments. (B) Logarithmic scale blotting of data shown in 1A. Specific fluorescence of each strain to the time of antibiotic addition was set to 100%. (C) Determination of half-lives via Western blot analysis of eYFP (27.0 kDa), eYFP-ASV (28.6 kDa) and eYFP-AAV (28.6 kDa). Cells were cultivated in 70 ml of BHI medium with 2% glucose to an OD_{600} of 3–4. Prior and after addition of Tet and Rif (addition of antibiotics after 1.5 h) 5 ml of cells were harvested by centrifugation and subsequently frozen in liquid nitrogen. For isolation of crude extract cells were ruptured with glass beads in TE buffer (10 mM Tris, 1 mM EDTA, pH 8) with complete protease inhibitor (Roche, Germany). Samples (25 µg) were loaded on two identical SDS gels and proteins were separated by SDS-PAGE and analysed via Western blot analysis using anti-GFP (cross-reacting to eYFP) and anti-lcd for referencing (80 kDa). The intensity of bands was analysed with the AIDA software version 4.15 (Raytest GmbH, Germany). The images are representative ones out of three independent biological replicates.

respective strains were inhibited by the addition of tetracycline and rifampicin (100 μg ml^{-1} and 250 μg ml^{-1}, respectively) to the culture medium. Both antibiotics were added 1.5 h after induction with IPTG. The decrease in fluorescence was measured in 10 min intervals in the BioLector system (Fig. 1A and B). In the course of these measurements, a stable signal was observed for native eYFP for > 24 h whereas a rapid decrease in signal was observed for eYFP-ASV and eYFP-AAV. The half-life of both variants was determined via Western blot analysis using anti-GFP antibodies cross-reacting towards eYFP. A rapid decrease in eYFP protein level was detected for both, ASV and AAV, tagged variants and half-lives of 22 ± 4 min (ASV) and 8 ± 3 min (AAV) were calculated (Fig. 1C and Table 2). The reference protein, isocitrate dehydrogenase (Icd), exhibited a stable signal in Western blot analysis within the period of measurement (Fig. 1C). These data are in agreement with studies of SsrA-tagged variants in *E. coli* or *Mycobacterium* species. In almost all cases ASV and AAV variants resulted in a moderate destabilization of reporter proteins, whereas the native tag and the LVA variant are very unstable and hardly useful for the study of expression kinetics (Andersen *et al.*, 1998; Triccas *et al.*, 2002; Blokpoel *et al.*, 2003; Allen *et al.*, 2007).

We also investigated the suitability of SsrA-tagged GFPuv variants as reporters for transient gene expression, since GFPuv has been used as an appropriate reporter for *C. glutamicum* promoters in previous studies (Knoppová *et al.*, 2007; Hänßler *et al.*, 2009). As observed for the eYFP variants, no signal was detectable for proteins fused to the native SsrA tag or the LVA version whereas for GFPuv tagged with either ASV or AAV, fluorescence could be monitored (Fig. 2). However, in contrast to eYFP, tagging of GFPuv led to an immense loss in fluorescent signal with a very low residual fluorescence of 10% (AAV) and 12% (ASV) compared with native GFPuv. Since these fluorescence levels were only slightly above background level, determination of reporter half-lives was hardly feasible (Fig. 2).

The results observed for eYFP and GFPuv show that the destabilizing effect conferred by a specific degradation tag depends very much on the protein it is fused to. In fact, when fusing the same SsrA tag variants (ASV and AAV) to the far-red dsRed derivative E2-Crimson (Strack *et al.*, 2009), only a slightly decreased protein stability was obtained, whereas a major fraction of the proteins seemed stable after blocking translation and transcription with antibiotics (data not shown). This effect might be due to the tetrameric structure of E2-Crimson masking the SsrA tag towards recognition by tail-specific proteases. A similar assumption was made by Allen and co-workers who constructed an SsrA-tagged version of the luciferase subunits LuxB and LuxA, respectively (Allen *et al.*, 2007). In their study, tagging of LuxB did not result in significant protein degradation; modification of both subunits, LuxA and LuxB, resulted in a rapid decay of bioluminescence. Therefore, a masking of the SsrA tag within a protein complex is an aspect which has to be considered for the destabilization of multimeric proteins.

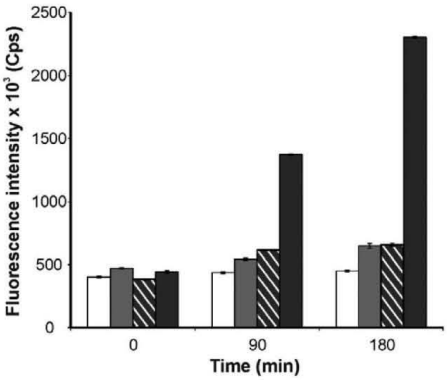

Fig. 2. Fluorescence of recombinant *C. glutamicum* strains expressing *gfp* variants. Shown is ATCC 13032/pEKEx2-*gfpuv* (black), ATCC 13032/pEKEx2-*gfpuv*-aav (patterned), ATCC 13032/pEKEx2-*gfpuv*-asv (grey) and ATCC 13032/pEKEx2 (white) as control. Cells were cultivated in CGXII minimal medium containing 1 mM IPTG to an OD_{600} of 3–5; 2 ml of cells were harvested by centrifugation and subsequently frozen in liquid nitrogen. Fluorescence was determined using Fluorolog 3 Double Spectrometer (Spex, USA). For this purpose, cells were thawed on ice, resuspended in 10 ml of cold TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) and the OD_{600} was determined. GFPuv fluorescence was carried out in triplicates using an excitation wavelength of 395 nm and recording emission at 509 nm. The graph shows the mean maximum fluorescence referred to the cell dry weight (1 ml of cell suspension of an OD_{600} of 1 corresponds to 0.36 mg of dry weight).

As proof of principle we applied the eYFP-ASV variant to study the dynamic expression of the *gntK* gene in *C. glutamicum*. Expression of *gntK*, encoding gluconate kinase, is stringently regulated by the transcriptional regulators GntR1 and GntR2 in response to carbon source availability (Frunzke *et al.*, 2008b). When gluconate, the substrate of GntK, is present, repression of *gntK* by GntR1/2 is relieved and expression of *gntK* is strongly induced in the exponential growth phase. To assess the suitability of the unstable eYFP-ASV variant, fusions of the P_{gntK} promoter with *eyfp* or *eyfp*-asv, respectively, were constructed and cloned into the vector pJC1. As expected, both promoter fusions gave rise to a significant fluorescent signal when the cells were cultivated in minimal medium containing gluconate as carbon source (Fig. 3A: 100 mM gluconate; Fig. 3B: 50 mM of glucose and gluconate). No signal was observed in minimal medium with 100 mM glucose (data not shown). In contrast to the reporter with native eYFP, the signal of the

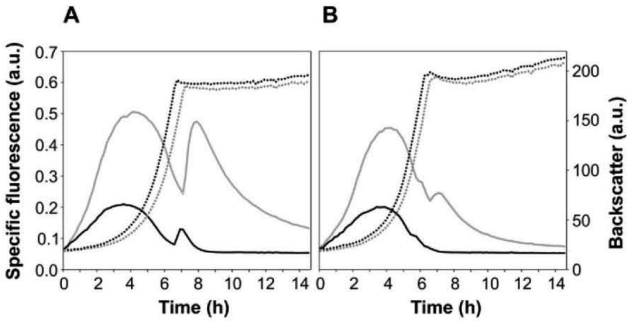

Fig. 3. Application of eYFP (grey) and destabilized eYFP-ASV (black) for dynamic gene expression analysis of *gntK* in *C. glutamicum*. Cells were inoculated to an OD_{600} of 1 in 750 µl of CGXII minimal medium containing either 100 mM gluconate (A) or 50 mM glucose and gluconate (B) in 48-well microtiter plates in the BioLector cultivation system (m2p labs, Germany). The final backscatter corresponds to a maximum OD_{600} of 35 and 38 for growth on 100 mM gluconate and 50 mM glucose plus gluconate, respectively. For pre-cultures, cells were cultivated in CGXII with 100 mM glucose. Growth (dashed line) and fluorescence (solid line) were recorded in 15 min intervals (for details see Fig. 1).

eYFP-ASV variant dropped back to zero when entering the stationary phase which is in agreement with enzyme activity measurements of GntK (Frunzke *et al.*, 2008b). The signal of native eYFP, however, did not reach background level within 24 h of measurement. This illustrates the suitability of unstable reporter variants to mirror the dynamic expression pattern of a gene of interest. Notably, the kink in specific fluorescence residing at the entrance into the stationary phase is due to delayed chromophore maturation in the log phase caused by oxygen limitation (Tsien, 1998; Shaner *et al.*, 2005; Drepper *et al.*, 2010). This effect is most likely less distinct for the unstable variant due to a lower amount of protein requiring oxygen for chromophore maturation. The kink in fluorescence was not observed with carbon source concentrations lower than 50 mM (data not shown). Interestingly, *C. glutamicum* expressing native *eyfp* showed a slight delay in growth in comparison with cells expressing the *eyfp*-asv variant. This indicates that expression of unstable reporter protein variants might even diminish the burden for the cell due to a rapid protein turnover and the avoidance of protein accumulation. A drawback of unstable variants is, however, the lowered reporter output (about twofold lower for eYFP-ASV) compared with the native reporter protein, which might lead to problems when monitoring genes with a low expression level. Consequently, the choice of the fluorescent protein variant, regarding spectral properties and protein half-life, clearly depends on the strength and dynamics of the promoter to be measured. Enlarging the tool box of reporter protein variants is required for optimal experimental design and output.

In a recent study, introduction of an SsrA-tagged variant of the enzyme TyrA into a phenylalanine producing *E. coli* strain was an elegant approach to improve the accumulation of phenylalanine (Doroshenko *et al.*, 2010). Our data reveal the applicability of corynebacterial SsrA tags and variants thereof for the efficient destabilization of eYFP and GFPuv in *C. glutamicum*. The use of SsrA peptide tagging is, yet, not limited to reporter proteins, but can be a valuable tool for the engineering of synthetic gene circuits (Elowitz and Leibler, 2000) or fine-tuning of protein levels in metabolic engineering of this important platform organism.

Conflict of interest

None declared.

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The two-component system ChrSA is crucial for haem tolerance and interferes with HrrSA in haem-dependent gene regulation in *Corynebacterium glutamicum*

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We recently showed that the two-component system (TCS) HrrSA plays a central role in the control of haem homeostasis in the Gram-positive soil bacterium *Corynebacterium glutamicum*. Here, we characterized the function of another TCS of this organism, ChrSA, which exhibits significant sequence similarity to HrrSA, and provide evidence for cross-regulation of the two systems. In this study, ChrSA was shown to be crucial for haem resistance of *C. glutamicum* by activation of the putative haem-detoxifying ABC-transporter HrtBA in the presence of haem. Deletion of either *hrtBA* or *chrSA* resulted in a strongly increased sensitivity towards haem. DNA microarray analysis and gel retardation assays with the purified response regulator ChrA revealed that phosphorylated ChrA acts as an activator of *hrtBA* in the presence of haem. The haem oxygenase gene, *hmuO*, showed a decreased mRNA level in a *chrSA* deletion mutant but no significant binding of ChrA to the *hmuO* promoter was observed *in vitro*. In contrast, activation from P_{hmuO} fused to *eyfp* was almost abolished in an *hrrSA* mutant, indicating that HrrSA is the dominant system for haem-dependent activation of *hmuO* in *C. glutamicum*. Remarkably, ChrA was also shown to bind to the *hrrA* promoter and to repress transcription of the paralogous response regulator, whereas *chrSA* itself seemed to be repressed by HrrA. These data suggest a close interplay of HrrSA and ChrSA at the level of transcription and emphasize ChrSA as a second TCS involved in haem-dependent gene regulation in *C. glutamicum*, besides HrrSA.

INTRODUCTION

Haem plays an important role as a cofactor for proteins of various functions and is used as an alternative source of iron by many bacterial species (Andrews *et al.*, 2003; Nobles & Maresso, 2011; Skaar, 2010). To ensure sufficient Fe^{2+} supply but also avoid toxic intracellular levels, iron uptake and utilization is usually tightly regulated at the transcriptional level (Andrews *et al.*, 2003; Hantke, 2001; Skaar, 2010). Classical two-component systems (TCSs), composed of a sensor histidine kinase and a cognate response regulator, represent a typical regulatory module to sense extracellular environmental stimuli and transduce the information via protein phosphorylation to the level of gene expression (Krell *et al.*, 2010; Mascher *et al.*, 2006; Stock *et al.*, 2000). Upon stimulus perception, the sensor kinase undergoes autophosphorylation of a conserved histidine residue; this phosphoryl group is subsequently transferred to an aspartate residue of the response regulator, which modulates gene expression by binding to the promoter region of target genes (Laub & Goulian, 2007; Stock *et al.*, 2000; West & Stock, 2001).

The Gram-positive soil bacterium *Corynebacterium glutamicum* represents an important platform organism in industrial biotechnology (Burkovski, 2008; Eggeling & Bott, 2005). In total, 13 TCSs are encoded in the *C. glutamicum* genome (Kocan *et al.*, 2006), several of which have been studied in more detail (Brocker *et al.*, 2011; Bott & Brocker, 2012; Schaaf & Bott, 2007; Schelder *et al.*, 2011). In a recent study, we demonstrated that the TCS HrrSA exhibits a central function in the control of haem homeostasis and haem utilization in *C. glutamicum*. In

Abbreviations: EMSA, electrophoretic mobility shift assay; TCS, two-component system; TSS, transcription start site.

Three supplementary tables and a more detailed method for the cloning techniques used here are available with the online version of this paper.

The normalized and processed microarray data from this study are available in the GEO database under accession no. GSF37327.

the presence of haem, the response regulator HrrA directly represses haem biosynthesis genes and activates haem oxygenase (*hmuO*) as well as genes encoding haem-containing components of the respiratory chain (Frunzke *et al.*, 2011). Expression of *hrrA* itself underlies control by the global iron regulator DtxR, which represses transcription from the promoter P_{hrrA}, downstream of *hrrS*, under conditions of sufficient iron supply (Wennerhold & Bott, 2006). Under iron-limiting conditions, DtxR dissociates from the *hrrA* promoter, thereby enabling the utilization of alternative iron sources such as haem. Besides *hrrA*, DtxR directly regulates the transcription of about 60 genes involved in iron uptake and storage in response to iron availability (Boyd *et al.*, 1990; Frunzke & Bott, 2008; Wennerhold *et al.*, 2005; Wennerhold & Bott, 2006).

For haem utilization, *C. glutamicum*, as well as its pathogenic relative *Corynebacterium diphtheriae*, depends on a haem uptake apparatus composed of the ABC transporter HmuTUV, several cell surface haem-binding proteins (Allen & Schmitt, 2009, 2011; Drazek *et al.*, 2000; Frunzke *et al.*, 2011) and a haem oxygenase (HmuO), which catalyses the intracellular degradation of the tetrapyrrol ring to α-biliverdin, free iron (Fe^{3+}) and carbon monoxide (Kunkle & Schmitt, 2007; Schmitt, 1997; Wilks & Schmitt, 1998). Acquisition of haem, however, exposes the respective organism to the toxicity associated with high levels of haem. It was shown in a recent study that the haem-regulated ABC transport system, HrtAB, is crucial for *C. diphtheriae* to cope with elevated haem concentrations (Bibb & Schmitt, 2010). The HrtAB system consists of the permease HrtB and the ATPase HrtA and is widespread among Gram-positive bacteria (Stauff *et al.*, 2008; Stauff & Skaar, 2009a, b). In *C. diphtheriae*, *hrtBA* expression was shown to be activated in the presence of haem by the TCS ChrSA (Bibb *et al.*, 2005; Bibb & Schmitt, 2010). In previous studies, the ChrSA system was described to activate expression of *hmuO* and repress expression of the *hemAC* operon encoding haem biosynthesis enzymes (Bibb *et al.*, 2007). Both targets, *hmuO* and *hemAC*, are also controlled by the second haem-dependent TCS, HrrSA, in *C. diphtheriae* (Bibb *et al.*, 2005, 2007).

Previous studies in *C. glutamicum* and *C. diphtheriae* revealed the TCSs HrrSA and ChrSA to have a global function in the control of haem homeostasis; however, no studies concerning the interplay of the two systems on the transcriptional level have been performed so far. In this report, we used genome-wide transcriptome analyses, protein–DNA interaction studies and promoter fusions to identify direct target genes of ChrSA (previously named CgtSR8) and study the interaction with the homologous system HrrSA in *C. glutamicum*. Our data reveal that HrrSA is the dominant system for the haem-dependent activation of haem oxygenase in *C. glutamicum*, whereas ChrSA plays a crucial role in haem tolerance mediated by the HrtBA haem transport system. Furthermore, we provide evidence for cross-regulation of both systems, HrrSA and ChrSA, at the level of transcription.

METHODS

Bacterial strains, media and growth conditions. The bacterial strains used in this study are shown in Table S1 (available with the online version of this paper). For growth experiments, a 20 ml preculture of CGXII minimal medium containing 4 % (w/v) glucose (Keilhauer *et al.*, 1993) was inoculated from a 5 ml BHI (brain heart infusion, Difco) culture after washing the cells with 0.9 % (w/v) NaCl. Cells were incubated overnight at 30 °C and 120 r.p.m. in a rotary shaker. The trace element solution with or without iron as well as the $FeSO_4$ or haemin (protoporphyrin IX with Fe^{3+}) solution were added from stock after autoclaving, as indicated. Standard CGXII minimal medium contains 36 µM $FeSO_4$. For the haemin stock solution, haemin (Sigma Aldrich) was dissolved in 20 mM NaOH to 250 µM. The main culture was inoculated from the second preculture to OD_{600} 1 in CGXII minimal medium containing 4 % (w/v) glucose and either $FeSO_4$ or haemin as iron source. For cloning purposes *Escherichia coli* DH5α was used; for overproduction of ChrA *E. coli* BL21(DE3) (Studier & Moffatt, 1986). *E. coli* was cultivated in Luria–Bertani (LB) medium at 37 °C or on LB agar plates. When necessary, kanamycin was added at an appropriate concentration (50 µg ml^{-1} for *E. coli* and 25 µg ml^{-1} for *C. glutamicum*). For growth experiments on agar plates the strains were grown in a 5 ml BHI culture overnight. The stationary culture was diluted to OD_{600} 1 and dilution series (3 µl each, 10^0 to 10^{-7}) were spotted on CGXII agar plates containing 4 % (w/v) glucose and either 2.5 or 36 µM $FeSO_4$ with or without haemin. Pictures of the plates were taken after incubation for 24 h at 30 °C.

Growth experiments in microtitre scale were performed in the BioLector system (m2p-labs). Therefore, 750 µl CGXII containing 2 % glucose (w/v) and different concentrations of $FeSO_4$ (2.5 or 36 µM) or haemin (2.5–20 µM) were inoculated with cells from a 20 ml CGXII preculture with iron-starved cells (0 µM $FeSO_4$) to OD_{600} 1 and cultivated in 48-well flowerplates (m2p-labs) at 30 °C, 1200 r.p.m. and a shaking diameter of 3 mm. The production of biomass was determined as the backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor of 10); measurements were taken in 10 min intervals. The average backscatter of non-growing wild-type cells (first 2 h of the wild-type in CGXII minimal medium with 15 µM haemin) was used for referencing. High fluctuations of low backscatter signals (non-growing cells, Fig. 1) are due to technical limitations. For promoter fusion studies, the eYFP chromophore was excited with 510 nm and emission was measured at 532 nm (signal gain factor of 50). The specific fluorescence (au) was calculated by the eYFP fluorescence signal per backscatter signal (Kensy *et al.*, 2009).

Cloning techniques. Routine methods were performed according to standard protocols (Sambrook *et al.*, 2001). Chromosomal DNA of *C. glutamicum* ATCC 13032 was prepared (Eikmanns *et al.*, 1994) and utilized as template for PCR. DNA sequencing and oligonucleotide synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Tables S1 and S2, respectively. A detailed description of the construction of strains and plasmids is given in the supplementary material.

DNA microarrays. The transcriptome of the deletion mutant Δ*chrSA* grown on haem or $FeSO_4$ was compared with the wild-type using whole-genome-based DNA microarrays. For this purpose, cells of a BHI preculture were used for inoculation of a second preculture in CGXII medium containing 1 µM $FeSO_4$. For main culture, cells were cultivated in CGXII minimal medium with 4 % glucose (w/v) containing either 2.5 µM $FeSO_4$ or haemin as iron source and harvested at OD_{600} 5–6 in pre-cooled (−20 °C) ice-filled tubes via centrifugation (6900 *g*, 10 min, 4 °C). The cell pellet was subsequently frozen in liquid nitrogen and stored at −70 °C until RNA

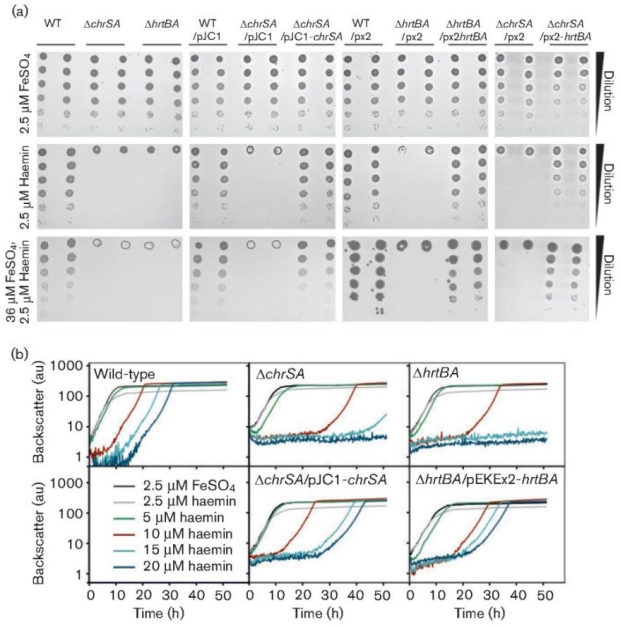

Fig. 1. Growth phenotype of *C. glutamicum* ATCC 13032 wild-type, Δ*chrSA* and Δ*hrtBA* mutants. (a) For growth on agar plates, cells were spotted on CGXII minimal medium plates in serial dilutions containing either 2.5 μM $FeSO_4$, 2.5 μM haemin or 36 μM $FeSO_4$ plus 2.5 μM haemin. (b) For growth in liquid culture, cells were resuspended in 750 μl CGXII minimal medium containing 2.5 μM $FeSO_4$ or haemin (2.5–20 μM) and cultivated in 48-well flowerplates in a BioLector system (see Methods). Growth was monitored as backscattered light (620 nm). Without iron, the cells reached a final backscatter value of about 50 (data not shown). Please note that the high fluctuations of backscatter values below 10 are due to technical limitations. Growth curves show one representative experiment of three biological replicates.

preparation. The preparation of total RNA was performed as described previously (Möker *et al.*, 2004). For cDNA synthesis, 25 ng total RNA from each sample was used. Labelling and hybridization was performed with a 70-mer custom-made DNA microarray purchased from Eurofins MWG Operon, as described previously (Frunzke *et al.*, 2008). All DNA microarray experiments were repeated in three biological replicates. The normalized and processed data were saved in the in-house microarray database (Polen & Wendisch, 2004) for further analysis and in the Gene Expression Omnibus (GEO) database under accession no. GSE37327.

Overproduction and purification of ChrA. For the overproduction of ChrA, *E. coli* BL21(DE3) was transformed with the vector pET28b-*chrA* and cultivated in 200 ml LB medium. At OD_{600} 0.6–0.8, the expression of *chrA* was induced by addition of 1 mM IPTG. After 4 h of expression at 30 °C, the cells were harvested by centrifugation (4000 ***g*** at 4 °C, 10 min). The cell pellet was stored at −20 °C until further use. For protein purification, the cell pellet was resuspended in 3 ml TNI5 buffer (20 mM Tris/HCl pH 7.9, 300 mM NaCl and 5 mM imidazole) containing Complete protease inhibitor cocktail (Roche). Cells were disrupted by passing through a French pressure cell (SLM Ainco, Spectronic Instruments) twice at 207 MPa. The cell debris was removed by centrifugation (6900 ***g***, 4 °C, 20 min), followed by ultracentrifugation of the cell-free extract for 1 h (150 000 ***g***, 4 °C). ChrA was purified from the supernatant via Ni^{2+}-NTA (nickel-nitriloacetic acid) affinity chromatography as described for *C. glutamicum* HrrA (Frunzke *et al.*, 2011). ChrA was eluted from the column with TNI100 buffer (containing 100 mM imidazole) and analysed on a 12 % SDS-polyacrylamide gel. Protein concentration was determined with Bradford reagent (Bradford, 1976). Elution fractions of ChrA were pooled and the buffer was exchanged to bandshift buffer [20 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM ATP, 10 mM $MgCl_2$, 5 % (v/v) glycerol, 0.5 mM EDTA, 0.005 % (w/v) Triton X-100] using a PD10 desalting column (GE Healthcare). The protein was stored in aliquots at −20 °C.

Electrophoretic mobility shift assay (EMSA). EMSAs were performed with purified ChrA protein and DNA fragments of the putative target genes. Promoter regions (500 bp) of the putative target genes were amplified via PCR and purified by using the Qiagen PCR purification kit. As a negative control, the promoter region of the *gntK* gene was used. DNA (100 ng per lane) was incubated with

different molar excesses of the purified ChrA protein at room temperature for 30 min in bandshift buffer. For phosphorylation of ChrA, 50 mM of the small phosphate donor phosphoramidate was incubated with the protein before the DNA was added. After incubation, sample buffer [0.1 % (w/v) xylene cyanol dye, 0.1 % (w/v) bromophenol blue dye, 20 % (v/v) glycerol in 1 × TBE (89 mM Tris base, 89 mM boric acid, 2 mM EDTA)] was added and samples were separated on a non-denaturing 10 % polyacrylamide gel with 170 V in 1 × TBE buffer. DNA was stained using SYBR Green I (Sigma-Aldrich). For verification of the ChrA binding motif, 30 bp double-stranded oligonucleotides were assembled by hybridization of two complementary oligonucleotides. The amount of shifted DNA was quantified by using the ImageQuant TL software (GE Heathcare).

Identification of transcription start sites (TSSs) and promoter regions by RNA-Seq. A 5′-end enriched RNA-Seq library was constructed according to the following procedures. 1) Depletion of stable rRNA and enrichment of mRNA molecules were performed using the Ribo-Zero rRNA removal kit for Gram-positive bacteria (Epicentre Biotechnologies). 2) The enriched mRNA was fragmented by magnesium oxaloacetate (MgKOAc) hydrolysis. Four vols RNA solution were mixed with one vol. MgKOAc solution (100 mM KOAc and 30 mM MgOAc in 200 mM Tris/HCl, pH 8.1) and the mixture was incubated for 2.5 min at 94 °C. The reaction was stopped by adding an equal volume of 1 × TE (10 mM Tris, 1 mM EDTA, pH 8) and chilling on ice for 5 min. 3) The fragmented RNA was precipitated by addition of three vols 0.3 M NaAc in ethanol with 2 µl glycogen and incubation overnight at −20 °C. 4) The precipitated RNA fragments were dissolved in water and the 5′-end RNA fragments were enriched by using Terminator 5′-phosphate-dependent exonuclease (Epicentre Biotechnologies). 5) After RNA precipitation (as above), the triphosphates were removed using RNA 5′-polyphosphatase (Epicentre Biotechnologies). 6) After RNA precipitation (as above), the 5′-enriched, monophosphorylated RNA fragments were used to construct a cDNA library by using the Small RNA Sample Prep kit (Illumina).

The fragmentation of RNA molecules (fragment sizes were 200–500 bp) and RNA concentration were monitored using the RNA 6000 Pico Assay on an Agilent 2100 Bioanalyser (Agilent). Sequencing of the cDNA library was carried out on the GA IIx platform (Illumina). Resulting reads were aligned to the *C. glutamicum* genomic sequence using the mapping software SARUMAN (Blom *et al.*, 2011). TSS and promoter regions were deduced by combining published information about promoter regions in *C. glutamicum* (Pátek & Nešvera, 2011) with 5′-end enriched RNA-Seq data.

RESULTS

The TCS ChrSA (previously CgtSR8): sequence similarities and genomic organization

In a previous study the TCS HrrSA was reported to play a central role in the control of haem homeostasis in *C. glutamicum* (Frunzke *et al.*, 2011). *In vitro* DNA binding studies with purified HrrA protein provided evidence that the response regulator HrrA binds to the upstream promoter region of an operon encoding another TCS, *chrSA* (cg2201–cg2200) (Kocan *et al.*, 2006). This system consists of the genes cg2200 (*chrA*, previously *cgtR8*), encoding the response regulator ChrA, and cg2201 (*chrS*, previously *cgtS8*), encoding the sensor kinase ChrS. Sequence analysis revealed considerable similarity of ChrSA to the recently described system HrrSA of *C. glutamicum*. The sensor kinases, ChrS and HrrS, share a sequence identity of about 35 %, whereas the response regulators, ChrA and HrrA, exhibit a sequence identity of about 58 % at the protein level (Table 1). Both systems also share significant similarities with HrrSA and ChrSA of *C. diphtheriae*. A pairwise comparison is given in Table 1. In terms of consistency with the previously described orthologous system of *C. diphtheriae*, we renamed CgtSR8 to 'ChrSA' for *C. glutamicum*.

RNA sequencing experiments indicated that, in contrast with the *hrrSA* operon, where a second promoter is located upstream of *hrrA*, the genes *chrSA* form a classical operon with one promoter upstream of *chrS* (Table S3). The start codon of *chrA* overlaps with the stop codon of *chrS*. Divergently from *chrSA* (intergenic region of 110 bp) the operon *hrtBA* is located, encoding the permease (HrtB) and ATPase (HrtA) components of an ABC-type transport system. Microsynteny is observed at this genomic locus consisting of a classical TCS and an operon encoding a 'haem-regulated transport system', which is highly conserved in Gram-positive bacteria. The transporter HrtAB has been described to be involved in export of haem or degradation products thereof (Stauff & Skaar, 2009a). These findings suggest that the TCS ChrSA might interfere in the control of haem homeostasis with the recently reported system HrrSA in *C. glutamicum*.

Deletion of *chrSA* leads to increased haem sensitivity

To characterize the role of the TCS ChrSA in haem utilization, we constructed an in-frame deletion mutant lacking the genes *chrA* and *chrS*. In first experiments, we analysed the haem tolerance of the deletion mutant Δ*chrSA* and the *C. glutamicum* wild-type. Growth of the strains was compared on agar plates or in liquid culture containing either haemin or $FeSO_4$ as iron source. Growth in liquid culture (2.5 µM $FeSO_4$ or 2.5–20 µM haemin) was performed in microtitre plates (48-well flowerplates, see

Table 1. Sequence identities of the TCSs ChrSA and HrrSA of *C. glutamicum* and *C. diphtheriae*

	Amino acid sequence identity (%)			
Response regulators	**1**	**2**	**3**	**4**
ChrA_Cg2200 (1)	100	–	–	–
ChrA_DIP2327 (2)	44	100	–	–
HrrA_Cg3247 (3)	58	52	100	–
HrrA_DIP2267 (4)	55	50	86	100
Sensor kinases	**5**	**6**	**7**	**8**
ChrS_Cg2201 (5)	100	–	–	–
ChrS_DIP2326 (6)	29	100	–	–
HrrS_Cg3248 (7)	35	25	100	–
HrrS_DIP2268 (8)	35	25	51	100

Methods) where *C. glutamicum* exhibits similar growth properties as in shake flasks.

When cultivated with $FeSO_4$ as an iron source, both wild-type and Δ*chrSA* showed the same growth phenotype on agar plates and in liquid minimal medium (Fig. 1). Grown on 2.5 μM haemin, Δ*chrSA* revealed a strong growth defect on plates (Fig. 1a). Under iron-replete conditions, the same phenotype was observed in the presence of haem (36 μM $FeSO_4$ and 2.5 μM haemin), indicating that the observed phenotype is a result of the elevated haem concentration and is not influenced by the iron concentration (Fig. 1a). In liquid culture, the presence of 2.5 μM haemin resulted in a decelerated growth rate and a lower final backscatter signal for both strains. The addition of 5 μM haemin extended the lag phase and resulted in a higher final backscatter compared with cells grown on 2.5 μM haemin, indicating that iron is a limiting factor under the chosen conditions. Higher haemin concentrations (10–20 μM) led to a proportional extension of the lag phase after which cells started to grow again with a growth rate comparable to cells grown on iron (Fig. 1b). Again, the mutant strain Δ*chrSA* exhibited a higher sensitivity towards elevated haemin concentrations (10–20 μM haemin). This delayed growth of the tested strains and the fact that the cells resume growth after the lag phase with an unaltered growth rate or final density led to the assumption that the added haemin is degraded in the culture medium over time until the concentration drops under a certain threshold. This tolerable limit would then be higher for the wild-type than for Δ*chrSA*. The observed phenotype of the Δ*chrSA* mutant was complemented by transformation with the plasmid pJC1-*chrSA*, expressing *chrSA* under the control of its native promoter (Fig. 1). Overall, these findings emphasize a central role of ChrSA in haem detoxification.

The HrtBA transporter confers resistance towards haem toxicity

Growth experiments revealed a significant haemin sensitivity of the Δ*chrSA* mutant. As outlined in the Introduction, the genes *hrtBA*, located divergently to *chrSA*, encode a putative 'haem regulated transporter' (Bibb & Schmitt, 2010; Stauff & Skaar, 2009b). Thus, a lowered expression of *hrtBA* in the Δ*chrSA* mutant could be a reason for the observed haem sensitivity of the Δ*chrSA* mutant. In order to investigate the function of the putative transport system HrtBA in *C. glutamicum*, an in-frame deletion mutant of the genes *hrtB* and *hrtA* was constructed. As observed for Δ*chrSA*, the growth of Δ*hrtBA* was not affected when $FeSO_4$ was added as sole iron source. In the presence of haemin, Δ*hrtBA* exhibited a significant growth defect, both on agar plates and during liquid cultivation (Fig. 1). This phenotype was complemented by transformation of Δ*hrtBA* with the plasmid pEKEx2-*hrtBA* carrying the *hrtBA* operon under the control of the IPTG-inducible P_{tac} promoter, which allows a basal gene expression even in the absence of IPTG. The strain Δ*hrtBA*/pEKEx2-*hrtBA* showed wild-type-like tolerance towards high haemin concentrations (Fig. 1). Induction of *hrtBA* expression by addition of IPTG led to a strong growth defect (data not shown). In the next step, we tested our hypothesis that reduced expression of *hrtBA* might be the reason for the observed growth phenotype of the Δ*chrSA* mutant and examined whether plasmid-driven expression of *hrtBA* in Δ*chrSA* could restore wild-type-like growth. In fact, the cross-complemented strain Δ*chrSA*/pEKEx2-*hrtBA* exhibited wild-type-like growth on agar plates containing 2.5 μM haemin (Fig. 1a). These data indicate that HrtBA plays a key function in haem detoxification in *C. glutamicum* and suggest a role of ChrSA in the control of *hrtBA* expression.

Transcriptome analysis of a Δ*chrSA* mutant strain

To identify additional potential target genes of ChrSA we assessed the influence of ChrSA on global gene expression via comparative transcriptome analysis of the Δ*chrSA* mutant and *C. glutamicum* wild-type grown in CGXII minimal medium with 4 % glucose and either 2.5 μM $FeSO_4$ or 2.5 μM haemin as iron source. Genes whose mRNA level showed a more than twofold alteration in either experiment ($FeSO_4$ or haemin) are listed in Table 2. In cells grown on $FeSO_4$, the deletion of *chrSA* had no significant influence on global gene expression. When cultivated with haemin as an iron source, the relative expression level of *hrtBA* (coding for the putative haem transport system HrtBA) was two- to threefold decreased in the Δ*chrSA* mutant.

Likewise, the expression of *hmuO*, encoding the haem oxygenase, was nearly sevenfold decreased in the presence of haemin, but showed no difference on iron as well. Expression of *hmuO* is also described as being under control of the global iron regulator DtxR in *C. glutamicum* (Wennerhold & Bott, 2006). In our studies, the Δ*chrSA* mutant showed a slightly reduced expression (1.3- to 2-fold) of several DtxR target genes (Table 2) composing the typical iron starvation response. Among those, we found the operon *hmuTUV* encoding a haem uptake system as well as *htaA*, *htaC* and *htaD* encoding putative haem-binding proteins. However, *hmuO* expression was significantly decreased even more than the other DtxR targets.

Remarkably, the mRNA level of *hrrA* encoding the response regulator of the TCS HrrSA was slightly increased (approx. 1.5-fold) in the Δ*chrSA* mutant. Together with the observed derepression of *chrSA* in a Δ*hrrA* mutant (Frunzke *et al.*, 2011) these data hint at a cross-regulation of both systems at the level of transcription. Further genes exhibiting an altered mRNA level include a regulator of unknown function (cg3303) and the redox-sensing regulator *qorR*, whose DNA-binding activity was reported to be affected by oxidants (Ehira *et al.*, 2009).

Identification of direct target genes of the response regulator ChrA

To test for direct binding of the response regulator ChrA to putative target promoters, we performed *in vitro* EMSA

Table 2. Comparative transcriptome analysis of Δ*chrSA* and *C. glutamicum* wild-type

This table shows all genes that revealed a ⩾ twofold altered relative mRNA (*P*-value ⩽0.06) level in at least two of three independent DNA microarrays of *C. glutamicum* Δ*chrSA* versus wild-type grown on CGXII minimal medium with 4 % (w/v) glucose and 2.5 μM $FeSO_4$ or haem as iron source.

Gene ID	Gene	Annotation	Ratio 2.5 μM $FeSO_4$*	Ratio 2.5 μM haem*
TCSs				
cg3247	*hrrA*	TCS, response regulator	1.03	1.45
cg3248	*hrrS*	TCS, signal transduction histidine kinase	1.01	0.86
Haem homeostasis-related genes				
cg2202	*hrtB*	ABC-type transport system, permease component	1.05	0.64
cg2204	*hrtA*	ABC-type transport system, ATPase component	1.17	0.33
cg2445	*hmuO*	Haem oxygenase	0.96	0.16
cg0466	*htaA*	Secreted haem transport-associated protein	0.97	0.48
cg0467	*hmuT*	Haemin-binding periplasmic protein precursor	0.89	0.68
cg0468	*hmuU*	Haemin transport system, permease protein	1.03	0.66
cg0469	*hmuV*	Haemin transport system, ATP-binding protein	0.98	n.d.
Others				
cg0018		Hypothetical membrane protein	1.02	2.02
cg1552	*qorR*	Redox-sensing transcriptional regulator	1.00	2.07
cg2518		Putative secreted protein	1.01	2.03
cg2845	*pstC*	ABC-type phosphate transport system, permease component	0.93	2.17
cg3303		Transcriptional regulator, PadR-like family	0.95	2.20

*The mRNA ratio represents the mean value of three independent DNA microarray experiments.

studies with purified ChrA. To this end, ChrA was overproduced in *E. coli* containing an N-terminal hexa-histidine tag and purified by affinity chromatography. Purified ChrA was phosphorylated by the addition of the small-molecule phosphate donor phosphoramidate, which led to an approximately two- to threefold increased affinity of ChrA~P to the tested DNA fragments.

In our assays, a clear binding of ChrA to the intergenic region of *chrSA* and *hrtBA* was detected (Fig. 2a). A complete shift was observed upon addition of a 30- to 50-fold molar excess of phosphorylated ChrA. Under these conditions neither the negative control (*gntK*, cg2732) nor the promoter region of *htaA* was bound by ChrA (data not shown). Binding of ChrA to a DNA fragment covering the promoter of *hrrA* was also observed, however, with a lower affinity than binding to *hrtBA–chrSA*. Notably, the promoter region of *hmuO* whose expression level was significantly decreased (sevenfold) in the Δ*chrSA* mutant was not bound by ChrA in this assay.

In further EMSA assays, the binding region of ChrA to the promoters of *hrtBA–chrSA* and *hrrA* was narrowed down to DNA fragments of about 30 bp. Positive sub-fragments covering the binding motif of ChrA showed a comparable shift from the originally tested fragments (Fig. 2b). For the *hmuO* promoter region EMSA assays with a subfragment covering the region upstream of the DtxR-binding region (−45 bp upstream of the TSS) showed a slightly different picture to the negative control, suggesting very low affinity binding of ChrA *in vitro*. Whether this binding is of physiological relevance has to be verified in further studies.

Mutational analysis of the ChrA-binding motif

Sequence analysis of the 30 bp DNA fragment in the intergenic region of *hrtBA* and *chrSA* revealed a small inverted repeat (CGACcaaaGTCG). To assess the relevance of this repeat for ChrA binding we performed mutational analysis of the whole 30 bp fragment. For this purpose, three to four nucleotides were exchanged for the complementary ones and the mutated fragments were tested in gel retardation analysis. The exchange of small inverted repeats abolished ChrA binding, whereas the exchange of adjacent nucleotides or the four nucleotides in between the repeat led to reduced ChrA affinity towards the particular DNA fragment (Fig. 3). Mutations outside of the motif did not affect ChrA binding. Overall, the mutational analysis supported the relevance of the inverted repeat for binding of ChrA and revealed the sequence AgTaCGACcaaaGTC-GgAtT as binding motif in the intergenic region of *hrtBA–chrSA*. A motif with considerable sequence identity was also found in the promoter region of *hrrA* (Fig. 4). A 30 bp fragment covering this predicted motif exhibited a clear binding by ChrA in EMSA assays (Fig. 2b).

Fig. 4 illustrates the position of the ChrA binding sites in relation to the TSS of the respective target gene. The TSS

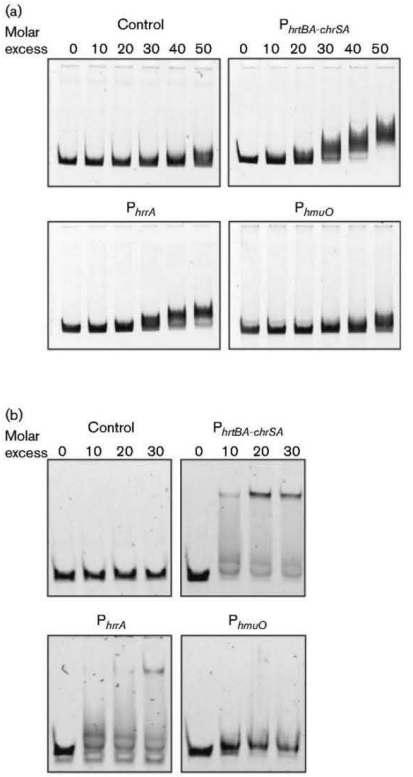

Fig. 2. DNA–protein interaction studies of ChrA and putative target promoters. (a) For gel retardation assays, 500 bp DNA fragments covering the promoter regions of *hrtBA–chrSA*, *hrrA* and *hmuO* were incubated without or with different molar excesses of phosphorylated ChrA (0- to 50-fold). The promoter region of *gntK* served as control fragment. For phosphorylation, purified ChrA protein was preincubated with 50 mM phopshoramidate (see Methods). Samples were separated on a 10% non-denaturing polyacrylamide gel and stained with SYBR green I. (b) As described in (a), 30 bp DNA fragments covering the putative binding site of ChrA. Samples were separated on a 15% non-denaturing polyacrylamide gel.

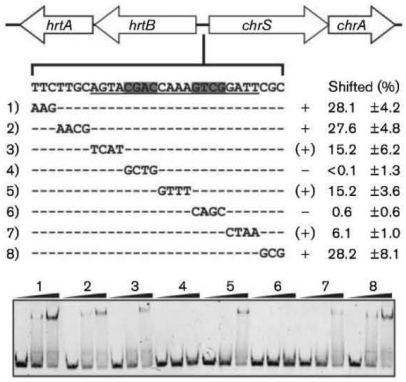

Fig. 3. Mutational analysis of the ChrA binding site in the intergenic region of *hrtBA–chrSA*. To analyse the relevance of different nucleotides for ChrA binding, a 30 bp DNA fragment covering the putative ChrA binding site in the *hrtBA–chrSA* intergenic region was mutated by an exchange of 3 to 4 bp to the complementary base pairs, as indicated, and analysed via EMSA studies. After incubation, the samples were separated on a 15% non-denaturing polyacrylamide gel and stained with Sybr Green I. +, Fragments that were shifted with unaltered affinity; (+), a shift with lower affinity; –, fragments that were not shifted. The amount of shifted DNA is given as a percentage and was quantified by using ImageQuant TL (GE Healthcare) from three experimental replicates (mean ± SD).

has been determined by RNA sequencing of the *C. glutamicum* transcriptome (see Table S3). In the *hrtBA–chrSA* intergenic region the ChrA motif is located in between the −35 regions of *hrtBA* and *chrSA*, a position that would be in agreement with ChrA having an activating function on the expression of both operons. In the case of *hrrA*, which showed a slightly increased mRNA level in the Δ*chrSA* mutant, the ChrA binding site is located close to the TSS and would support a proposed repressor function of ChrA interfering at this locus with the binding of the RNA polymerase (Madan Babu & Teichmann, 2003).

HrrSA and ChrSA interfere in haem-dependent gene regulation

Previous studies revealed binding of the response regulator HrrA to the *hrtBA–chrSA* intergenic region. In view of the data reported in this study, HrrA and ChrA likely interfere in the transcription control of *hrtBA* and/or *chrSA*. To study the influence of both TCSs *in vivo* we constructed promoter fusions of the intergenic region of *hrtBA–chrSA* fused to *eyfp* in both possible directions (P_{chrSA} and P_{hrtBA}). While the wild-type containing the reporter plasmids (WT/pJC1-P_{hrtBA}-*eyfp*, WT/pJC1-P_{chrSA}-*eyfp*) exhibited no fluorescence when grown on iron, cells grown on haem showed a significantly increased fluorescence signal in the lag and early exponential phase (Fig. 5a). The Δ*chrSA* strain transformed with the promoter fusion plasmids (Δ*chrSA*/pJC1-P_{hrtBA}-*eyfp*, Δ*chrSA*/pJC1-P_{chrSA}-*eyfp*) showed no

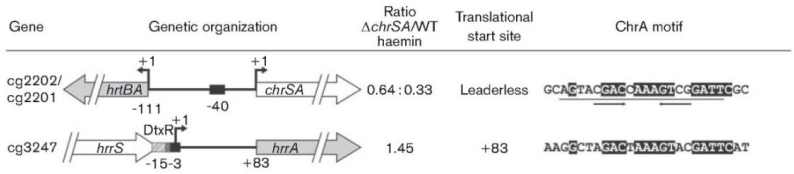

Fig. 4. Localization of ChrA binding sites in the *hrtBA–chrSA* intergenic region and the *hrrA* promoter. Promoters were derived from RNA sequencing experiments; the corresponding −10 and −35 regions are given in Table S3. The TSS is indicated as +1 and the ChrA binding sites are shown as a black box, the DtxR binding site is shown as shaded box. The number below the ChrA box indicates the distance to the TSS. The mRNA ratios were obtained from DNA microarray analysis [Δ*chrSA* mutant versus wild-type (WT) grown on 2.5 μM haemin, see also Table 2]. Nucleotides conserved in both motifs are shaded in black.

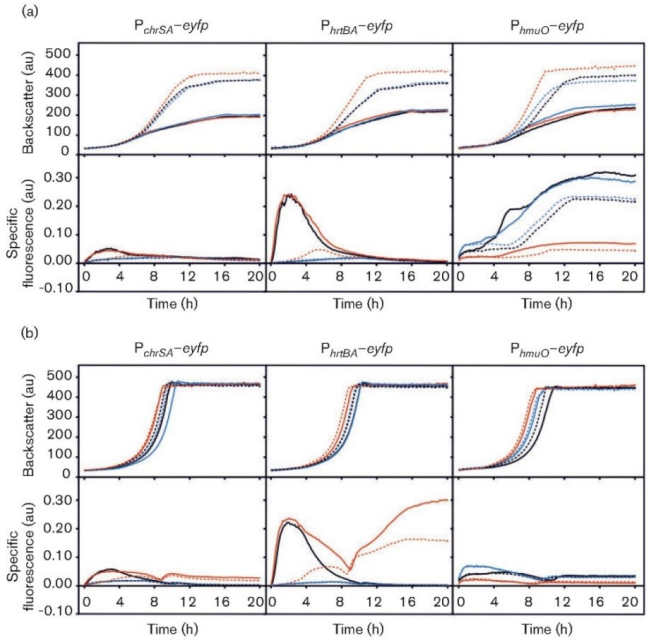

Fig. 5. Promoter studies of P_{chrSA}, P_{hrtBA} and P_{hmuO} in wild-type and TCS mutants. For promoter studies, the promoters of *chrSA*, *hrtBA* and *hmuO* were fused to *eyfp*. C. *glutamicum* wild-type (black), Δ*chrSA* (blue), and Δ*hrrSA* (red) were cultivated in CGXII minimal medium with 2 % glucose in microtitre plates (a) with 2.5 μM $FeSO_4$ (dotted lines) or 2.5 μM haemin (solid lines) as iron source or (b) with 36 μM $FeSO_4$ with (solid lines) or without (dotted lines) 2.5 μM haemin. In the BioLector system, the growth (backscatter signal of 620 nm light) and eYFP fluorescence (excitation 510 nm/emission 532 nm) were monitored over 10 min intervals. The specific fluorescence was calculated as fluorescence signal per backscatter signal (given in arbitrary units, au). Shown are representative experiments of three to four independent replicates.

significant fluorescent signal (Fig. 5a), indicating that ChrSA is crucial for the haem-dependent activation of both promoters. A similar response was observed under iron-excess conditions (36 μM $FeSO_4$) in the presence of haem (Fig. 5b). These data are in line with a positive autoregulation of *chrSA* and a ChrA-dependent activation of *hrtBA* in haem-grown cells. In a Δ*hrrSA* strain, lacking the genes *hrrA* and *hrrS* of the TCS HrrSA, a higher signal was detected for both promoters (P_{hrtBA} and P_{chrSA}) under iron limitation in comparison with the wild-type, supporting the postulated repressor function of HrrA on *chrA* (Fig. 5). Remarkably, under iron-replete conditions, the activity of P_{hrtBA} and P_{chrSA} in the Δ*hrrSA* strain remained high throughout the exponential and stationary growth phase and did not decline to the background level.

HrrSA is crucial for the haem-dependent activation of *hmuO*

Transcriptome analysis of the Δ*chrSA* mutant revealed a significant reduction of the *hmuO* mRNA level in the mutant strain when cultivated on haem. However, no significant binding of ChrA was observed to the *hmuO* promoter. In the following experiment we used a promoter fusion of P_{hmuO} to *eyfp* to further study the impact of the TCSs HrrSA and ChrSA on *hmuO* expression *in vivo*. In contrast with P_{hrtBA}-*eyfp* and P_{chrSA}-*eyfp* whose expression peaked in the early exponential phase, the wild-type containing the P_{hmuO}-*eyfp* construct showed an increasing signal during the exponential phase in cells grown on haem as an iron source. In iron-grown cells (2.5 μM $FeSO_4$), *hmuO* expression showed an increase later in the mid-exponential phase, which correlates with the derepression of the iron starvation response controlled by DtxR (Wennerhold & Bott, 2006). Under iron-replete conditions (36 μM $FeSO_4$) the activity of P_{hmuO} was reduced to an almost background level (Fig. 5b). In the absence of *chrSA*, a similar course was observed during cultivation on iron, whereas the increase of P_{hmuO} activity on haem-grown cells was slightly delayed in the exponential phase (Fig. 5a) but reached wild-type levels in the stationary phase. Remarkably, the fluorescent signal was strongly diminished in a mutant lacking *hrrSA* (Δ*hrrSA*/pJC1-P_{hmuO}-*eyfp*). These data emphasize a central role of HrrSA in haem-dependent activation of *hmuO* expression in *C. glutamicum*.

DISCUSSION

Many bacterial species rely on haem or haem proteins as alternative sources of iron. Here, we showed that the TCS ChrSA is the crucial regulatory system for resistance towards haem toxicity in the non-pathogenic soil bacterium *C. glutamicum*. We identified the putative haem exporter *hrtBA* and *hrrA*, which encodes the response regulator HrrA of the homologue TCS HrrSA, as direct target genes of the response regulator ChrA. The highest binding affinity of purified ChrA was observed in the presence of the phosphate donor phosphoramidate, indicating that ChrA follows the classical model and is active in its phosphorylated state (Gao *et al.*, 2007; Stock *et al.*, 2000). This is consistent with recent studies where the phosphotransfer from the soluble kinase domain of ChrS to the response regulator ChrA was described for the *C. diphtheriae* ChrSA system (Burgos & Schmitt, 2012). The autophosphorylation of ChrS was shown to occur in the presence of haemin in purified *E. coli* proteoliposomes, indicating a direct interaction of ChrS with haem (Ito *et al.*, 2009).

The results described in this study support the prediction that *C. glutamicum* ChrSA has a key function in activating the expression of the divergently located operon *hrtBA* in the presence of haem. In fact, this function of ChrSA was expected due to the conserved microsynteny of this genomic locus where an operon of a TCS system is found in divergent orientation to *hrtBA* encoding a putative 'haem-regulated' ABC-transport system. This genomic organization is highly conserved among Gram-positive bacteria and homologous HrtAB transport systems were described as being required for coping with toxic haem concentrations for the species *C. diphtheriae*, *Staphylococcus aureus* and *Bacillus anthracis* (Bibb & Schmitt, 2010; Stauff *et al.*, 2008). So far, this transport system has mainly been described in pathogenic species where it is of major importance during host infection, when the bacteria are exposed to high haem concentrations in the blood. The presence of *hrtBA* in the genome of the non-pathogenic soil bacterium *C. glutamicum* might be a relic of evolution, as *C. glutamicum* is closely related to several pathogenic *Corynebacteria*, such as *C. diphtheriae* or *Corynebacterium ulcerans* (Yukawa *et al.*, 2007). However, high haem tolerance might also be of benefit in the soil, where haem is present in decaying organic material and represents an attractive alternative iron source for aerobic bacteria (Andrews *et al.*, 2003). An alternative regulatory mechanism of transcriptional regulation of *hrtBA* has recently been reported for the Gram-positive commensal bacterium *Lactococcus lactis*. Here, the cytoplasmic one-component regulator HrtR was described as a crucial factor for the haem-dependent activation of *hrtBA* (Lechardeur *et al.*, 2012). This mechanism is conserved among different Gram-positive commensals and contrasts with the TCS-mediated control described for several pathogenic species as well as *C. glutamicum*.

By using gel retardation assays and mutational analysis, we identified an imperfect inverted repeat (AgTaCGACcaaaG-TCGgAtT) as a ChrA binding site within the *hrtBA–chrSA* intergenic region. Five of the eight nucleotides composing the inverted repeat are conserved in the binding site in the *hrrA* promoter. A genome-wide motif search did not reveal candidates for additional, putative binding sites of haem-relevant genes, probably due to the poor conservation of the motif. The motif revealed only weak similarities to the identified ChrA binding motif upstream of *C. diphtheriae hrtBA* (CatATCAACCagtcGGTTGATggG) or with the motif of the ChrA orthologues HssR from *S. aureus* and *B.*

anthracis (Bibb & Schmitt, 2010; Burgos & Schmitt, 2012; Stauff & Skaar, 2009b). However, our data reveal differences in the network composition of *C. glutamicum* ChrSA and HrrSA in comparison to what is known for the *C. diphtheriae* systems. An adequate example therefore is the haem-dependent gene regulation of the haem oxygenase (*hmuO*). Whereas ChrSA was reported to be the prominent system involved in haem-dependent *hmuO* activation in *C. diphtheriae* (Bibb *et al.*, 2005, 2007), promoter fusion studies in this work emphasize HrrSA to hold this function in *C. glutamicum*, since almost no signal of the P_{hmuO}-*eyfp* construct was observed in a Δ*hrrSA* mutant. Reduced *hmuO* expression was also observed in a Δ*chrSA* mutant by transcriptome analysis, but promoter fusion studies suggested that this effect might rather be due to delayed *hmuO* expression in the early and mid-exponential phase. Whether this effect is directly mediated by ChrA is currently unclear as no significant binding of ChrA to the *hmuO* promoter was observed in our *in vitro* studies. A potential binding motif that shares slight similarity to the *C. diphtheriae* motif is located upstream of the DtxR binding site in the *hmuO* promoter (−45: TCCAACTAAGGGACTA). A binding motif for HrrA has not been reported so far but binding of HrrA is also likely to be located in this promoter region (upstream of −35). As the two response regulators ChrA and HrrA share significant sequence identity (62 % sequence identity in the DNA-binding helix–turn–helix motif), it can be speculated whether both regulators might bind to overlapping or even identical regions with different affinities – a question which will be addressed in following studies.

Although the HrrSA and ChrSA systems share high sequence similarity, the genomic organization differs. In contrast with *chrSA*, *hrrA* expression is repressed by the global iron regulator DtxR under iron sufficiency and is derepressed when iron becomes limiting (Wennerhold & Bott, 2006) (Fig. 6). This control of *hrrA* by DtxR seems not to be present in *C. diphtheriae* (Bibb *et al.*, 2007). Variations in the regulatory network composition in these closely related species may be surprising; however, sequence analysis revealed striking differences between *Corynebacterium* species regarding their TCS equipment (Bott & Brocker, 2012). With respect to HrrSA and ChrSA, several corynebacterial genomes contain only one of the two systems; both systems together were identified in the *C. glutamicum* species *C. diphtheriae*, *C. pseudotuberculosis* and *C. lipophiloflavum* (Bott & Brocker, 2012; Cerdeño-Tárraga *et al.*, 2003; Kalinowski *et al.*, 2003; Trost *et al.*, 2010; Yukawa *et al.*, 2007). These findings indicate significant variation at the species level and suggest an individual network constitution meeting the requirements of each particular species.

The fact that both TCSs HrrSA and ChrSA are involved in haem-dependent gene regulation already suggests that the two systems might interact with each other. Here, we provided evidence for a cross-regulation of HrrSA and ChrSA at the level of transcription. In our previous studies, we observed a weak binding of HrrA to the intergenic region of *hrtBA–chrSA* (Frunzke *et al.*, 2011). This result is further

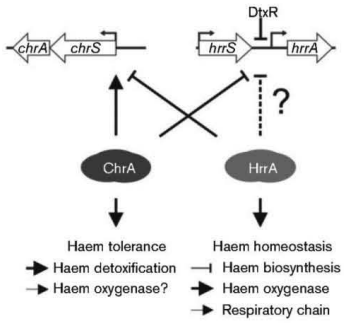

Fig. 6. The current model of ChrSA and HrrSA cross-regulation in *C. glutamicum*. Based on current insights, ChrSA is the prominent system required for dealing with haem toxicity and activates *hrtBA* expression in the presence of haem. Under iron limitation, *chrSA* is repressed by HrrA to avoid haem export when haem is required as an alternative iron source. As a balancing counterpart, *hrrA* itself, but not *hrrS*, is repressed by ChrA. Altogether, the current results emphasize a high level of regulatory network linkage to balance haem detoxification and the use of haem as a protein cofactor and alternative iron source.

supported by the finding that expression from P_{chrSA} is increased in a Δ*hrrSA* mutant indicating repression of *chrSA* by the homologous system (Fig. 5). This effect is especially obvious under iron limitation where HrrSA seems to be the dominating system ensuring additional iron supply from haem by the activation of haem oxygenase (Fig. 5). Additionally, our data suggest haem-dependent repression of *hrrA* by ChrA. Our current model shown in Fig. 6 emphasizes that this cross-regulation acts as a balancing act to avoid toxic levels on the one hand and ensure iron acquisition on the other hand. Remarkably, this cross-regulation only affects the expression of *hrrA* and not *hrrS*, which seems to be expressed at a constitutively low level; no significant difference in the level of *hrrS* mRNA was observed in transcriptome comparisons of Δ*chrSA* and wild-type. A further level of interaction was suggested in previous studies of the *C. diphtheriae* systems, which provided evidence for *in vivo* cross-talk by phosphoryl transfer of HrrSA and ChrSA (Bibb *et al.*, 2005, 2007). Altogether, these data provide striking evidence for a close link between the HrrSA and ChrSA systems and further studies are required to understand the interplay between these TCSs and the physiological relevance thereof.

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Phosphatase activity of the histidine kinases ensures pathway specificity of the ChrSA and HrrSA two-component systems in *Corynebacterium glutamicum*

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Summary

The majority of bacterial genomes encode a high number of two-component systems controlling gene expression in response to a variety of different stimuli. The Gram-positive soil bacterium *Corynebacterium glutamicum* contains two homologous two-component systems (TCS) involved in the haem-dependent regulation of gene expression. Whereas the HrrSA system is crucial for utilization of haem as an alternative iron source, ChrSA is required to cope with high toxic haem levels. In this study, we analysed the interaction of HrrSA and ChrSA in *C. glutamicum*. Growth of TCS mutant strains, *in vitro* phosphorylation assays and promoter assays of P_{hrtBA} and P_{hmuO} fused to *eyfp* revealed cross-talk between both systems. Our studies further indicated that both kinases exhibit a dual function as kinase and phosphatase. Mutation of the conserved glutamine residue in the putative phosphatase motif DxxxQ of HrrS and ChrS resulted in a significantly increased activity of their respective target promoters (P_{hmuO} and P_{hrtBA} respectively). Remarkably, phosphatase activity of both kinases was shown to be specific only for their cognate response regulators. Altogether our data suggest the phosphatase activity of HrrS and ChrS as key mechanism to ensure pathway specificity and insulation of these two homologous systems.

Introduction

Two-component systems (TCS) represent a prevalent way in which bacteria sense a variety of different extra- and intracellular stimuli and transduce this information to the level of gene expression (Capra and Laub, 2012). The prototypical type of a TCS is comprised of a membrane-bound histidine kinase and a cytoplasmic response regulator. Upon reception of a specific stimulus, the histidine kinase undergoes autophosphorylation at a conserved histidine residue. Phosphotransfer to the aspartate residue of a cognate response regulator typically elicits an adjustment of gene expression and adaptation of the physiology to the particular environmental conditions (Stock *et al.*, 2000; Laub and Goulian, 2007). Often dozens of these systems are found within one bacterial species and have evolved to respond to a variety of different signals including nutrients, changes in redox state or osmolarity, and antibiotics. The presence of a large number of related signal transduction systems, however, raises the question of how distinct pathways are insulated to prevent unwanted cross-talk and to permit specific signal transduction (Podgornaia and Laub, 2013).

In previous studies we reported on two homologous TCS, HrrSA and ChrSA, which play a central role in the control of haem homeostasis in the Gram-positive soil bacterium *Corynebacterium glutamicum* (Eggeling and Bott, 2005; Bott and Brocker, 2012). We demonstrated that HrrSA plays an important role in the utilization of haem as an alternative iron source in this important biotechnological model organism. In the presence of external haem, the response regulator HrrA activates the expression of the *hmuO* gene coding for the haem oxygenase, and of genes encoding haem-containing components of the respiratory chain (Frunzke *et al.*, 2011). The haem oxygenase is required for the utilization of haem as an alternative iron source as it catalyses the cleavage of the tetrapyrrol ring structure resulting in the release of iron (Wilks and Schmitt, 1998). At the same time, HrrA acts as a repressor of genes coding for haem biosynthesis enzymes. The expression of *hrrA* itself and of its target gene *hmuO* is repressed by DtxR, the key regulator of iron homeostasis which is conserved in many bacterial species, thereby ensuring the preferential utilization of free iron (Schmitt and Holmes, 1991a,b; Wennerhold and Bott, 2006). In *C. glutamicum* DtxR controls the transcription of about 60 genes involved in iron uptake and storage in response to the iron availability (Wennerhold *et al.*, 2005; Wennerhold and Bott, 2006; Frunzke and Bott,

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2008). A tight control of iron homeostasis is crucial for almost all living species to ensure sufficient supply of this important trace element but to avoid high intracellular levels of Fe(II), catalysing the formation of reactive oxygen species (Pierre and Fontecave, 1999; Andrews *et al.*, 2003).

Recently, the ChrSA system was described representing a second TCS involved in haem-dependent control of gene expression in *C. glutamicum*. ChrSA is crucial for conferring resistance against high levels of haem and shares high sequence similarity with the HrrSA system in *C. glutamicum* (HrrS and ChrS: 39%; HrrA and ChrA: 57% identity, Fig. 1) and *Corynebacterium diphtheriae* (Bibb and Schmitt, 2010; Heyer *et al.*, 2012). ChrSA directly activates the divergently located operon *hrtBA* that encodes a putative haem exporter, which is conserved in various Gram-positive species, e.g. *C. diphtheriae*, *Bacillus anthracis*, and *Staphylococcus aureus* (Stauff *et al.*, 2008; Stauff and Skaar, 2009; Bibb and Schmitt, 2010).

The coexistence of two TCSs in one species, which share a high sequence similarity and are both involved in haem-dependent gene regulation, raises the question if and on which levels these systems interact with each other. Due to the high sequence similarity of the HrrSA and ChrSA systems there is potential for considerable cross-talk at the level of phosphorylation. Laub and co-workers defined the term 'cross-talk' as an unwanted interaction between TCS (e.g. as a result of the deletion of a particular component) and cross-regulation as beneficial cross-phosphorylation of a particular signal transduction

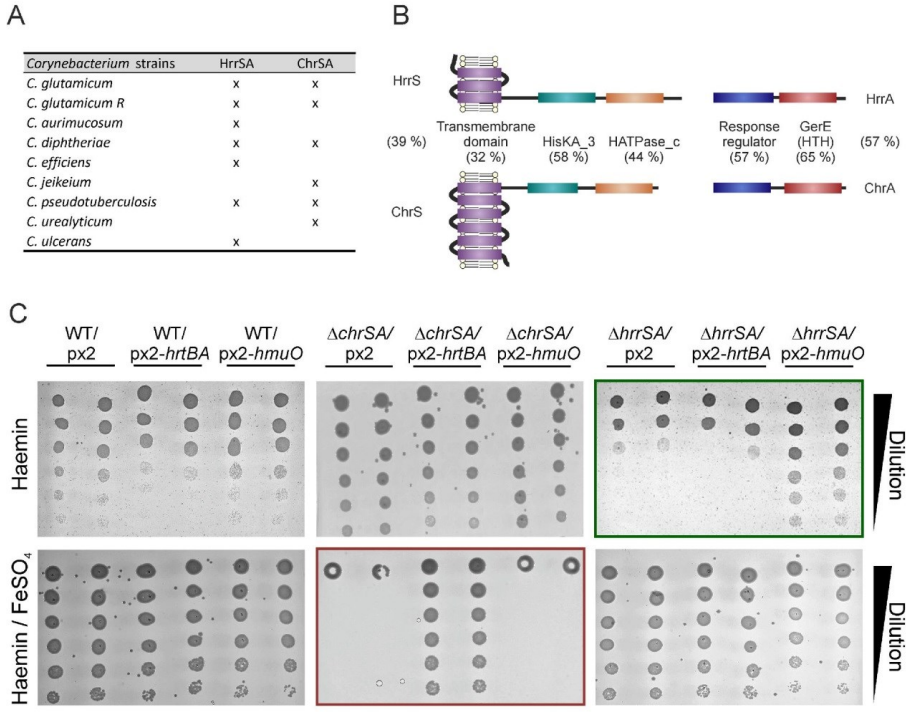

Fig. 1. HrrSA and ChrSA in *Corynebacterium*.
A. The distribution of the TCS HrrSA and ChrSA in different *Corynebacterium* species was obtained from Bott and Brocker (2012).
B. Identity of ChrSA and HrrSA TCS. Sequence analysis was performed using NCBI BLAST, domain architecture was obtained from Pfam and transmembrane prediction was performed with HMMTOP and TopPredII.
C. Discrimination of growth phenotypes of the *C. glutamicum* Δ*chrSA* and Δ*hrrSA* mutants. The strains harboured the indicated plasmids (px2, px2-*hrtBA*, or px2-*hmuO*). For growth on agar plates cells were spotted in serial dilutions on CGXII glucose minimal medium plates containing 1 mM IPTG and either 2.5 μM haemin (–PCA, strong iron limitation) or 2.5 μM $FeSO_4$ and 2.5 μM haemin (+PCA, iron available). Plates were incubated at 30°C for 48 h.

pathway. Only a few studies proving the physiological relevance of cross-regulation have been described in literature so far (Laub and Goulian, 2007; Podgornaia and Laub, 2013). Reporter studies with *C. diphtheriae* target promoters provided first evidence of cross-talk at the level of phosphotransfer between the ChrSA and HrrSA systems (Bibb *et al.*, 2007). In order to regulate the level of phosphorylated response regulator or to prevent undesired cross-talk, most histidine kinases exhibit bimodal function both as kinase and as phosphatase on their cognate response regulators (Laub and Goulian, 2007; Huynh *et al.*, 2010; Kenney, 2010; Huynh and Stewart, 2011; Willett and Kirby, 2012; Podgornaia and Laub, 2013).

Here, we present a comprehensive analysis addressing the specificity of signal transduction of the ChrSA and HrrSA systems and the interaction of these two systems in haem-dependent gene regulation in *C. glutamicum*. Activity profiling of ChrSA and HrrSA target promoters revealed specific signal transduction of the two homologous systems in the wild type whereas considerable cross-talk in mutant strains lacking one of the two sensor kinases was observed. Mutational analysis and reporter studies demonstrated phosphatase activity of the two sensor kinases HrrS and ChrS and substrate competition as crucial mechanisms ensuring pathway specificity and insulation in *C. glutamicum*.

Results

Phenotyping of HrrSA and ChrSA mutants

The two TCS HrrSA and ChrSA are highly conserved among different *corynebacterial* species. Whereas some species harbour only one of the TCS, in the genome of, for example, *C. glutamicum* ATCC 13032, *C. glutamicum* R, *C. diphtheriae* and *Corynebacterium pseudotuberculosis* both TCS can be found (Fig. 1A and Fig. S2). In *C. glutamicum* both TCS share a high sequence identity (Fig. 1B and Fig. S2). Prior studies of the two TCS ChrSA and HrrSA revealed both of them to play an important role in the control of haem homeostasis in *C. glutamicum*. Whereas the ChrSA system is crucial for activating the expression of the divergently located operon *hrtBA*, encoding a putative haem export system, it was shown that the HrrSA system is responsible for the activation of the haem oxygenase (*hmuO*) (Frunzke *et al.*, 2011; Heyer *et al.*, 2012). Growth experiments revealed an increased sensitivity of both, *chrSA* and *hrrSA*, mutant strains towards haem. However, no significant difference of growth phenotypes as indication for separate signal transduction pathways was disclosed so far. As a starting point of our work, we set out to adjust growth conditions in such a way that we were able to discriminate between mutations in the *hrrSA* and *chrSA* genes. In fact, a severe growth defect of the *C. glutamicum* Δ*chrSA* strain, but not of the Δ*hrrSA* strain, was observed on agar plates containing CGXII minimal medium with 2.5 μM haem and 2.5 μM $FeSO_4$ [plus the iron chelator protocatechuic acid (PCA)]. This growth defect could be restored by plasmid-based expression of the main target operon *hrtBA* (Fig. 1C, red box). Under these conditions, no growth defect of *hrrSA* mutants was observed (Fig. 1C). When the availability of free iron was significantly reduced (–$FeSO_4$, –PCA) and only haem was offered as alternative iron source, the growth of the Δ*hrrSA* mutant was significantly affected (Fig. 1C, green box). The wild type phenotype could be restored by plasmid-based expression of the main target gene *hmuO* (Fig. 1C, green box) or by expression of *hrrSA* with the plasmid pJC1-*hrrSA* (Fig. S1). The Δ*chrSA* mutant showed wild type-like growth under these conditions (+haem, –PCA). Expression of *hmuO* in Δ*chrSA* and *hrtBA* in Δ*hrrSA* as well as the empty vector control led to no significant recovery of growth (Fig. 1C). On standard minimal medium containing 36 μM $FeSO_4$ all mutants grew like the wild type strain (data not shown).

These differences in mutant phenotypes indicated that both systems inherit different roles in the control of haem homeostasis and provided a first starting point for the systematic dissection of HrrSA and ChrSA to study pathway specificity and interaction of these systems in *C. glutamicum*.

Activity profiling of the hmuO and hrtBA promoters

In the following, we analysed the response of the HrrSA and the ChrSA systems to iron (0–36 μM) and/or haem (0–8 μM) availability by using promoter fusions of the main target promoters P_{hmuO} and P_{hrtBA} to the reporter gene *eyfp*. The promoter activities were recorded by means of the specific eYFP fluorescence (fluorescence per biomass) of the reporter strains containing either pJC1-P_{hrtBA}-*eyfp* or pJC1-P_{hmuO}-*eyfp* (Fig. 2). For a direct comparison of the tested conditions, the average fluorescence maximum (8 h for P_{hmuO} and 2.5 h for P_{hrtBA} respectively) was determined (Fig. 2C and D). To monitor the influence of the ChrSA or HrrSA system on their respective target promoter, the reporter signal of the respective deletion mutants (almost background in both cases) was subtracted from the wild type signal (Fig. 2E and F).

Monitoring of the P_{hmuO}-*eyfp* reporter activity revealed an increasing fluorescence signal with rising haem concentrations in the absence of iron ($FeSO_4$ < 1 μM) in wild type cells. The eYFP fluorescence increased about sevenfold from 0 μM to 8 μM haem. Higher haem concentrations affected the growth of *C. glutamicum* wild type and were therefore avoided in this assay. In the presence of iron, however, the P_{hmuO} promoter activity strongly declined to almost background level (Fig. 2A). This is in

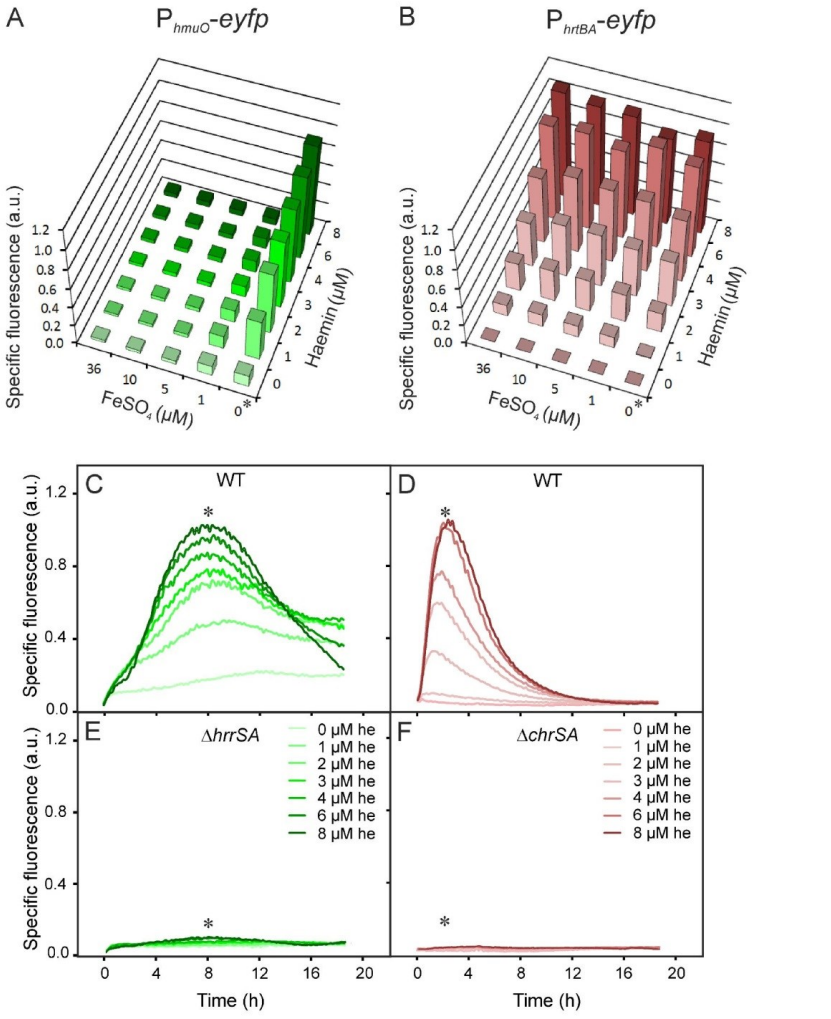

Fig. 2. Activity profiling of *hrtBA* and *hmuO* promoters. Strains carrying the reporter plasmids pJC1-P_{hrtBA}-*eyfp* and pJC1-P_{hmuO}-*eyfp* were cultivated in the BioLector system in CGXII minimal medium with 2% (w/v) glucose in microtitre plates with different haem (0–8 µM) and $FeSO_4$ (0–36 µM) concentrations. $FeSO_4$ was added together with PCA to promote efficient uptake. Growth (backscatter signal of 620 nm light) and eYFP fluorescence (excitation 510 nm/emission 532 nm) were monitored. The specific fluorescence was calculated as fluorescence signal per backscatter signal (given in arbitrary units, a.u.).
A. The P_{hmuO} activity of the wild type (WT) recorded after 8 h is shown; the background fluorescence of the Δ*hrrSA* deletion strain was subtracted from the WT signal.
B. The P_{hrtBA} activity recorded after 2.5 h in the WT is presented; the background fluorescence of the Δ*chrSA* deletion strain was subtracted from the WT signal.
C–F. Cells were cultivated with different haem (he) concentrations (0–8 µM, –PCA). P_{hmuO} (C, E) and P_{hrtBA} (D, F) activity was measured in 10 min intervals for 19 h in the WT (C, D), Δ*hrrSA* (E) and Δ*chrSA* deletion strains (F).
Asterisks indicate the chosen maxima that are presented in panels A and B. Representative experiments of three independent biological replicates are shown.

agreement with the fact that *hrrA* and *hmuO* expression is repressed by the master regulator of iron homeostasis, DtxR, in the presence of iron, thereby allowing the preferential utilization of $FeSO_4$ as iron source (Wennerhold and Bott, 2006; Frunzke *et al.*, 2011).

In contrast to the P_{hmuO}-*eyfp* reporter, activation of the P_{hrtBA}-*eyfp* reporter was only dependent on haem availability (Fig. 2B). Independent of the $FeSO_4$ supply, the P_{hrtBA}-*eyfp* signal increased with rising haem concentrations. From 0 μM haem to 8 μM haem we observed an about 130-fold increase of eYFP fluorescence in the absence of iron after 2.5 h (Fig. 2D). In the absence of haem (0 μM) no activation of the P_{hrtBA}-*eyfp* reporter could be detected after 2.5 h (Fig. 2B). These data indicate a strict pathway specificity of these two systems in *C. glutamicum*.

ChrA and HrrA are phosphorylated by the histidine kinases ChrS and HrrS

To test whether phosphorylation of the response regulators ChrA and HrrA is exclusively mediated by the histidine kinases HrrS and ChrS, we first performed growth experiments of a mutant (Δ*hrrS*/Δ*chrS*) lacking the genes of both kinases. In comparison to the wild type the Δ*hrrS*/Δ*chrS* mutant exhibited a growth defect when cultivated in the presence of haem, irrespective of the absence or presence of $FeSO_4$ (Fig. 3A). In a second approach, P_{hmuO}-*eyfp* and P_{hrtBA}-*eyfp* reporter output was monitored in the Δ*hrrS*/Δ*chrS* mutant in the presence of 2.5 μM haem (+PCA). The activity of both reporters was almost reduced to background level in the Δ*hrrS*/Δ*chrS* mutant and showed a 4- (P_{hmuO}) and 25-fold (P_{hrtBA}) decreased signal in comparison to the wild type (Fig. 3B). Mutant strains, in which the codon for the conserved aspartate residue D54 of HrrA or ChrA had been exchanged chromosomally by an alanine codon (WT::HrrA-D54A and WT::ChrA-D54A), also exhibited an increased sensitivity towards haem (Fig. 3A) and a similar output of the P_{hmuO}-*eyfp* and P_{hrtBA}-*eyfp* reporters as the Δ*hrrS*/Δ*chrS* mutant (Fig. 3B). These data suggested that the residues HrrA-D54 and ChrA-D54 are crucial for transcriptional activation of *hmuO* and *hrtBA*, respectively, and that probably no other sensor kinases beside HrrS and ChrS and also no small-molecule phosphoryl donors, such as acetyl phosphate, can efficiently phosphorylate the response regulators HrrA and ChrA *in vivo*.

In vivo *cross-talk of the ChrSA and HrrSA systems*

In order to test for a cross-talk of the HrrSA and ChrSA systems, a set of single and double deletion mutants was constructed (Table 1). In line with the results described above, deletion of the whole *hrrSA* operon, of the response regulator gene *hrrA* alone, or of both kinase genes *hrrS* and *chrS*, led to a significant growth defect (Fig. 4A) of the respective strains on haem plates under iron limitation (−PCA). Remarkable, a mutant lacking only the *hrrS* gene exhibited wild type-like growth behaviour (Fig. 4A, green box). Since no external $FeSO_4$ was added,

Table 1. Bacterial strains and plasmids used in this study.

Strains	Characteristics	Reference
Escherichia coli		
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* (DE3)	Studier and Moffatt (1986)
C. glutamicum		
ATCC13032	Biotin-auxotrophic wild type	Kinoshita *et al.* (2004)
Deletion mutants	In-frame deletion of operons/genes	
ATCC13032 Δ*hrrSA*	*hrrSA* (cg3248, cg3247)	Heyer *et al.* (2012)
ATCC13032 Δ*chrSA*	*chrSA* (cg2201, cg2200)	Heyer *et al.* (2012)
ATCC13032 Δ*hrrS*	*hrrS* (cg3248)	This work
ATCC13032 Δ*hrrA*	*hrrA* (cg3247)	Frunzke *et al.* (2011)
ATCC13032 Δ*chrS*	*chrS* (cg2201)	This work
ATCC13032 Δ*chrA*	*chrA* (cg2200)	This work
ATCC13032 Δ*hrrS*/Δ*chrS*	*hrrS* (cg3248) and *chrS* (cg2201)	This work
ATCC13032 Δ*chrS*/Δ*hrrA*	*chrS* (cg2201) and *hrrA* (cg3247)	This work
Deletion mutants	Mutation of phosphorylation site: amino acid exchange of aspartate to alanine at position 54	
ATCC13032 *hrrA*-D54A	HrrA Asp54 to Ala	This work
ATCC13032 *chrA*-D54A	ChrA Asp54 to Ala	This work
ATCC13032 Δ*chrS*/Δ*hrrA-chrA*-D54A	ChrA Asp54 to Ala	This work
Deletion mutants	Amino acid exchange of catalytic residue in the phosphatase domain (glutamine to alanine)	
ATCC13032 *hrrS*-Q222A	HrrS Gln222 to Ala	This work
ATCC13032 *chrS*-Q191A	ChrS Gln191 to Ala	This work

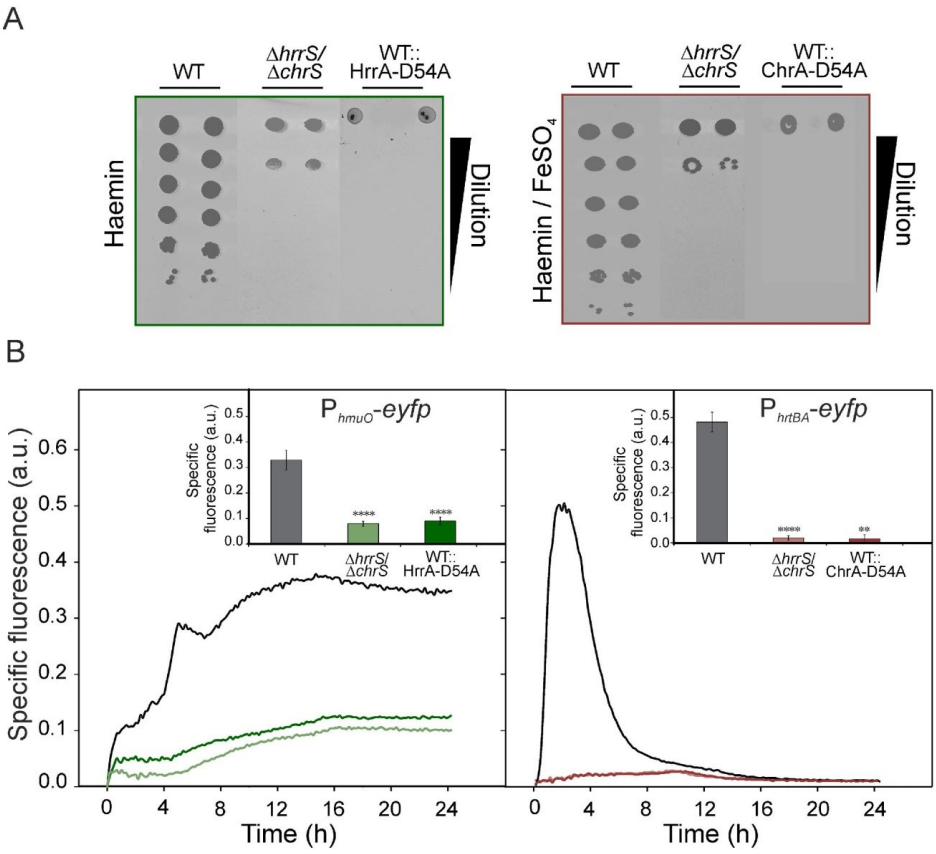

Fig. 3. The kinases ChrS and HrrS are crucial for the activation of ChrA and HrrA.
A. Growth of the *C. glutamicum* ATCC 13032 wild type (WT), Δ*hrrS*/Δ*chrS*, WT::HrrA-D54A and WT::ChrA-D54A on CGXII agar plates. Cells were spotted in serial dilutions on plates containing either 2.5 μM haemin (–PCA) or 2.5 μM $FeSO_4$ and 2.5 μM haemin (+PCA).
B. P_{hmuO} activity (left) was measured in *C. glutamicum* ATCC 13032 wild type (WT) (grey), Δ*hrrS*/Δ*chrS* (light green) and WT::HrrA-D54A (dark green). P_{hrtBA} activity (right) was measured in the WT (grey), Δ*hrrS*/Δ*chrS* (light red) and WT::ChrA-D54A (dark red). Strains carrying the reporter plasmids pJC1-P_{hrtBA}-*eyfp* and pJC1-P_{hmuO}-*eyfp* were cultivated in the BioLector system in CGXII minimal medium with 2% (w/v) glucose in microtitre plates with 2.5 μM haem (+PCA). eYFP fluorescence was monitored in 10 min intervals. P_{hmuO}-*eyfp* was recorded after 8 h and for P_{hrtBA}-*eyfp* after 2.5 h (means of three independent replicates). Asterisks show significant differences calculated by Student's unpaired *t*-test ($n = 3$; $^{**}P < 0.01$; $^{****}P < 0.0001$).

degradation of haem by the haem oxygenase HmuO is required to utilize haem as alternative source of iron for growth. To determine *hmuO* expression, the P_{hmuO}-*eyfp* reporter was transferred into the different mutant strains. When cultivated with haemin as sole iron source, the Δ*hrrSA*, Δ*hrrA* and Δ*hrrS*/Δ*chrS* mutants showed an almost fivefold reduced signal compared to the wild type. Remarkably, the Δ*hrrS* mutant exhibited a ~ 2.5-fold higher signal compared to the strains Δ*hrrSA*, Δ*hrrA*, and Δ*hrrS*/Δ*chrS* (Fig. 4A), suggesting cross-talk of HrrA by the ChrS sensor kinase in this genetic background. As expected, the deletion of *chrS* resulted in a wild type-like growth and *hmuO* expression under the tested conditions (Fig. 4A).

In the presence of bioavailable iron (+PCA), cultivation on haem plates revealed a growth defect of the Δ*chrSA*,

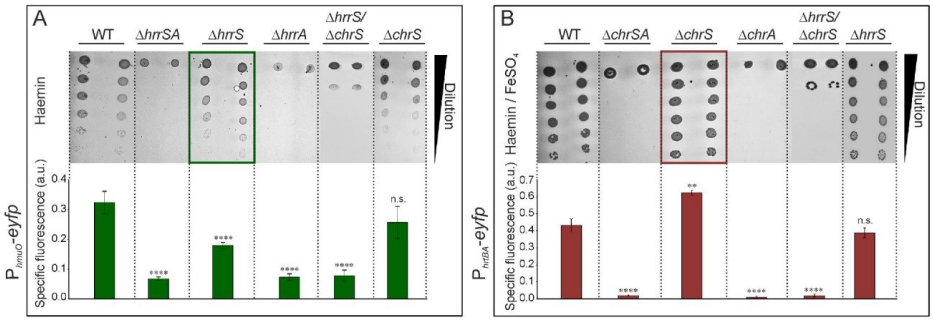

Fig. 4. Cross-talk between HrrSA and ChrSA.
A. The growth phenotypes of mutant strains suggest that ChrS is able to phosphorylate HrrA *in vivo* (CGXII plates with 2.5 μM haemin, −PCA). Fluorescence of mutant strains containing the P_{hmuO}-*eyfp* reporter was recorded after 8 h cultivation in the BioLector system.
B. Vice versa, growth of mutants revealed that HrrS is able to phosphorylate ChrA *in vivo* (CGXII plates with 2.5 μM haemin and 2.5 μM $FeSO_4$, +PCA). Fluorescence of strains containing the P_{hrtBA}-*eyfp* reporter was recorded after 2.5 h cultivation in the BioLector system.
Results are means of three independent replicates. Asterisks show significant differences calculated by Student's unpaired *t*-test ($n = 3$; n.s., not significant; $^{**}P < 0.01$; $^{****}P < 0.0001$).

Δ*chrA* and Δ*hrrS*/Δ*chrS* mutants. Under these conditions, activation of the *hrtBA* operon is required to maintain a low intracellular haem level (Fig. 4B). This was also confirmed by reporter assays with the P_{hrtBA}-*eyfp* reporter. Strains lacking *chrSA*, *chrA*, or both kinase genes (*hrrS* and *chrS*) showed no significant fluorescence signal (Fig. 4B). Remarkably, a strain lacking the histidine kinase ChrS displayed a wild type-like growth behaviour (Fig. 4B, red box) and the fluorescent output of the P_{hrtBA}-*eyfp* reporter in this mutant was even 1.4-fold increased in comparison to the wild type signal (Fig. 4B). These data corroborate a cross-talk of ChrA by HrrS in the Δ*chrS* background. Note that also deletion of *hrrS* did not affect growth behaviour or activation of P_{hrtBA}-*eyfp* in comparison to the wild type (Fig. 4B).

In vitro *phosphotransfer*

To confirm cross-talk of the HrrSA and ChrSA systems *in vitro*, the cytoplasmatic kinase domains of HrrS and ChrS as well as the response regulators HrrA and ChrA were overproduced in *Escherichia coli* BL21(DE3) and purified by affinity chromatography (see *Experimental procedures*). As a control, the sensor kinase and response regulator of the PhoSR TCS were used in this study (Schaaf and Bott, 2007). To initiate autophosphorylation, kinases were incubated with [γ-^{33}P]-ATP and samples were taken as indicated and analysed via SDS-PAGE and autoradiography. After addition of the respective response regulators, a phosphotransfer from ChrS to ChrA and HrrA could already be detected after 30 s, whereas no phosphotransfer to the negative control PhoR could be observed within 60 min (Fig. 5A). Quantification revealed a preference of ChrS phosphorylating ChrA. In this assay, the maximal intensity of the ChrA~P band was obtained after 2.5 min, whereas phosphorylation of HrrA by ChrS was slightly delayed (Fig. 5B).

Also in the case of HrrS, an immediate phosphotransfer to HrrA and ChrA was observed 30 s after the addition of response regulators, whereas no phosphotransfer to PhoR was detected (Fig. 5A). Again, a kinetic preference of HrrS phosphorylating HrrA was observed. Maximal phosphorylation was obtained after 1 min for HrrA and after 5 min in the case of ChrA (Fig. 5B). In a control assay, no phosphotransfer from PhoS to HrrA or ChrA was observed, but solely the cognate response regulator PhoR was phosphorylated by PhoS *in vitro* (Fig. 5A). Together with the growth phenotypes of the mutant strains and the reporter studies described in the previous section, these *in vitro* assays corroborate the possibility of cross-talk between the HrrSA and ChrSA TCS.

Evidence for a dual function of ChrS as kinase and phosphatase of ChrA

In order to evade a putative cross-talk between different TCS, the phosphorylation state of response regulators is tightly controlled. Besides their kinase function, many histidine kinases have been reported to exhibit a dual function as kinase and phosphatase of their cognate response regulator (Kenney, 2010; Willett and Kirby, 2012). Interestingly, we observed a significant growth defect of a Δ*chrS*/Δ*hrrA* mutant when cultivated on standard CGXII minimal medium containing 2.5 μM $FeSO_4$ as iron source (Fig. 6A). None of the other TCS mutants displayed a phenotype under this particular condition. In fact, we

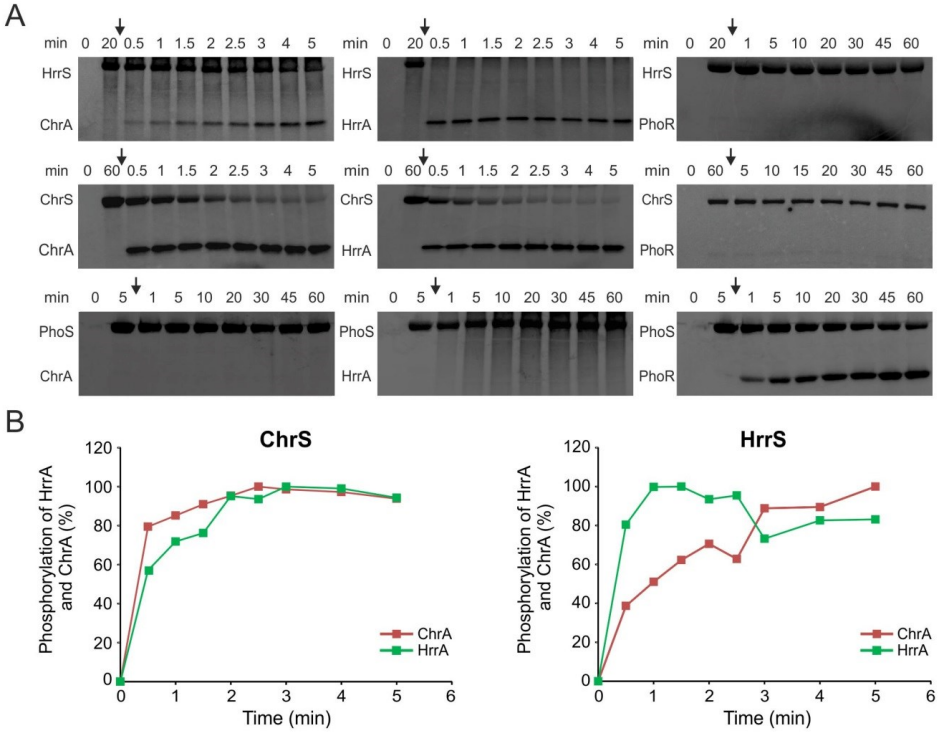

Fig. 5. *In vitro* phosphotransfer from the kinases MBP-ChrSΔ1–176, MBP-HrrSΔ1–169, and MBP-PhoSΔ1–246 to the response regulators HrrA-NHis, ChrS-NHis, and PhoR-NHis.
A. Purified MBP-kinases (6 μM final concentration) were incubated with [γ-^{33}P]-ATP and purified response regulators were added (12 μM final concentration) as indicated by arrows. The samples were incubated at room temperature for a further 60 min. At the indicated times, samples were taken, mixed with SDS loading buffer, and stored on ice (see *Experimental procedures*). Samples were separated by SDS-PAGE and dried for 1 h at 80°C. A storage phosphor screen (Fuji Imaging Plates BAS-MS, 20 × 25 cm) was exposed to the dried gel for ~ 20 h and the screen was analysed with a Typhoon Trio Scanner (GE Healthcare, Germany).
B. Quantification in relation to maximum phosphorylated protein was performed with the ImageQuant TL software (GE Healthcare).

observed a more than 20-fold increased P_{hrtBA} activity in the Δ*chrS*/Δ*hrrA* strain in comparison to the wild type when cells were cultivated either with haem or with iron for 2.5 h (Fig. 6B). In contrast to the wild type, the reporter signal did not decline to background level in the mutant strain (data not shown). This growth defect of the Δ*chrS*/Δ*hrrA* mutant could be reversed by an amino acid exchange of the phosphorylated D54 residue of ChrA, indicating that (hyper-)phosphorylated ChrA causes the observed phenotype. A mutant lacking only the *chrS* gene also showed a significantly increased reporter output on haem (Fig. 6B) and also no drop to background level could be observed when cultivated on haem or iron (Fig. 6C). Based on these findings, we speculated that a hyperactivity of the ChrA response regulator due to a lacking ChrS phosphatase activity leads to an overexpression of *hrtBA* in the Δ*chrS*/Δ*hrrA* mutant. The export of *de novo* synthesized haem might be the reason for the observed growth defect shown in Fig. 6A.

Phosphatase activity of ChrS and HrrS ensures pathway specificity

Amino acid sequence analysis revealed a conserved putative phosphatase motif (DxxxQ) in the HisKA_3 domains of both, ChrS and HrrS (Fig. 6E), which was recently described to be crucial for the phosphatase activity of NarX in *E. coli* (Huynh *et al.*, 2010). To test for a

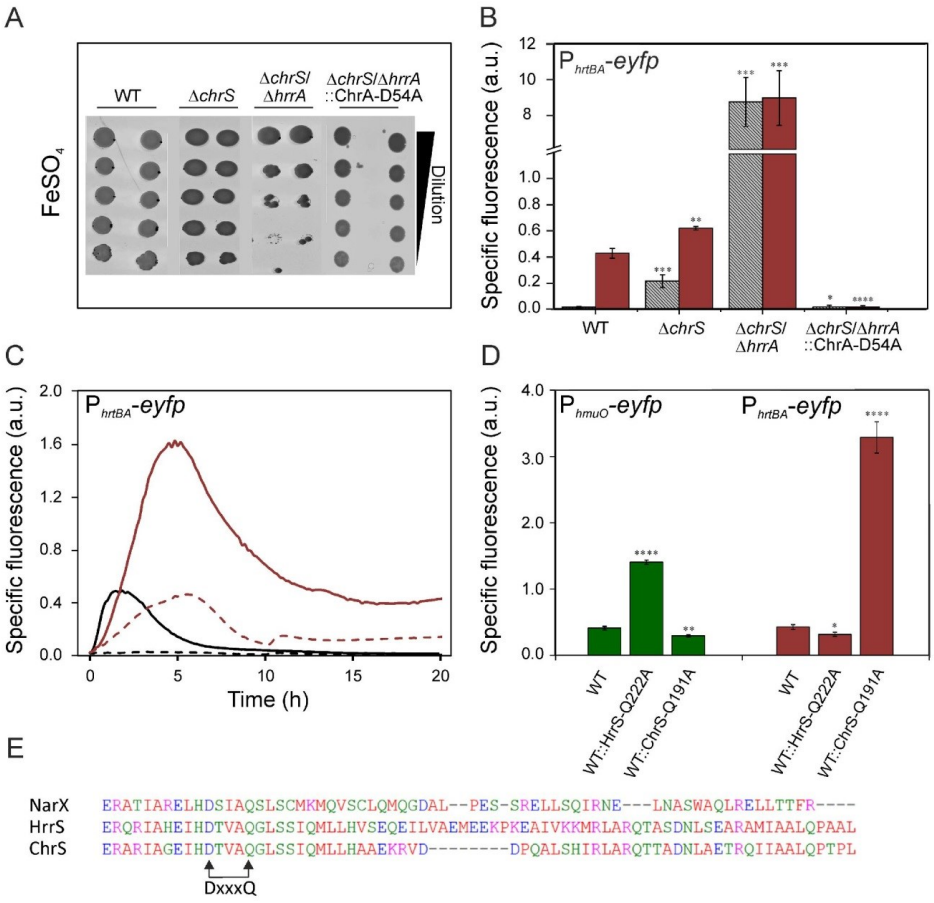

Fig. 6. Phosphatase activity of HrrS and ChrS is required for pathway specificity.

A. Cells of the indicated strains were spotted in serial dilutions on CGXII plates containing 2.5 μM $FeSO_4$ (+PCA) and were incubated at 30°C for 48 h.

B. Deletion of *chrS* leads to a strongly increased activity of the P_{hrtBA}-*eyfp*; eYFP fluorescence is even higher in a strain lacking *chrS* and *hrrA*. Strains carrying the reporter plasmid pJC1-P_{hrtBA}-*eyfp* were cultivated in the BioLector system in CGXII minimal medium with 2.5 μM $FeSO_4$ (+PCA) (shaded) or 2.5 μM haemin (+PCA) (red) and eYFP fluorescence was recorded after 2.5 h ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

C. P_{hrtBA}-*eyfp* fluorescence of the WT strain (black) and the Δ*chrS* mutant (red). Strains carrying the reporter plasmid pJC1-P_{hrtBA}-*eyfp* were cultivated for 20 h as described above in CGXII medium containing 2.5 μM haemin (solid lines) or 2.5 μM $FeSO_4$ (dashed lines).

D. Mutation of the putative phosphatase motif DTVAQ of HrrS and ChrS to DTVAA results in an upregulation of their particular target promoters P_{hmuO} and P_{hrtBA} respectively. Strains carrying the reporter plasmid pJC1-P_{hrtBA}-*eyfp* or pJC1-P_{hmuO}-*eyfp* were cultivated as described above and eYFP fluorescence was recorded after 12 h ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$).

E. Alignment of the amino acid sequence of the HisKA_3 domains of the histidine kinases NarX from *E. coli* K-12 and HrrS and ChrS from *C. glutamicum* ATCC 13032. The DxxxQ phosphatase motif is highlighted by arrows.

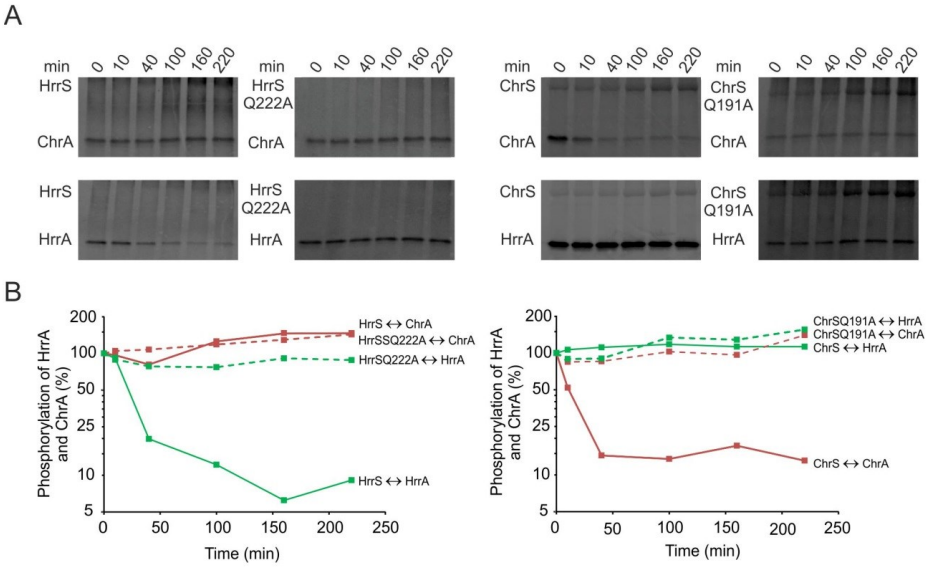

Fig. 7. Determination of *in vitro* phosphatase activity of HrrS and ChrS and phosphatase OFF muteins.
A. Purified kinases (ChrSΔ1–176, HrrSΔ1–169, ChrS-Q191AΔ1–176 and HrrS-Q222AΔ1–169) (6 μM final concentration) were incubated with [γ-^{33}P]-ATP and purified response regulators were added (12 μM final concentration) as indicated and incubated at room temperature. After 20 min ($t = 0$) samples were taken and processed as described in the legend of Fig. 5.
B. Quantification was performed as described in Fig. 5.

putative phosphatase function, the conserved glutamine residue in the DxxxQ motif was exchanged to alanine by suitable codon exchanges in the chromosomal *chrS* and *hrrS* genes of the wild type. When transformed with the P_{hmuO}-*eyfp* reporter, the HrrS-Q222A mutant exhibited a 1.4-fold increased signal of P_{hmuO} in comparison to the wild type (Fig. 6D). In contrast, a Q191A exchange in ChrS did not affect P_{hmuO} activity. Vice versa, a ChrS-Q191A mutant displayed a nearly eightfold increased P_{hrtBA}-*eyfp* reporter signal, whereas no effect on P_{hrtBA} was observed in the HrrS-Q222A background (Fig. 6D). This indicates that phosphatase activity of HrrS and ChrS might be specific only for their cognate response regulators.

To analyse the specificity of phosphatase activity, *in vitro* phosphorylation experiments with purified native components (as described above) and phosphatase OFF muteins (HrrS-Q222A-MalE and ChrS-Q191A-MalE) were performed. Remarkably, bimodal function of HrrS as kinase and phosphatase could only be observed for its cognate response regulator HrrA (Fig. 7A left). For HrrA a decrease of the phosphorylation of about 94% could be observed within 160 min whereas ChrA showed a constant phosphorylation level (Fig. 7B left). The phosphatase mutant HrrS-Q222A was not able to dephosphorylate neither HrrA nor ChrA (Fig. 7A and B left). Vice versa, phosphatase activity of ChrS was observed only for the cognate response regulator ChrA (Fig. 7A right). The phosphorylation level dropped about 85% within 40 min whereas phosphorylation of HrrA remained constant during the experiment (Fig. 7B right). Mutation of the conserved glutamine residue resulted in a more or less complete loss of phosphatase activity of both muteins: ChrS-Q191A and HrrS-Q222A (Fig. 7A and B right). These results emphasize that the phosphatase activity of HrrS and ChrS might be crucial to ensure pathway specificity of these homologous TCS and that the conserved glutamine residue in the DxxxQ motif is essential for phosphatase activity of both sensor kinases.

Discussion

The genome of *C. glutamicum* encodes 13 TCS which are all of the prototypical type consisting of a membrane-bound sensor kinase and a cytoplasmic response regulator (Bott and Brocker, 2012). Remarkably, this non-pathogenic soil bacterium expends two of these systems to control gene expression in dependence of external haem availability. In agreement with previous studies, the

results of the present study confirm two distinct roles of HrrSA and ChrSA in the control of haem homeostasis in *C. glutamicum*. Whereas the HrrSA system inherits the function to mediate utilization of haem as an alternative source of iron by activating the expression of the haem oxygenase gene *hmuO* (Frunzke *et al.*, 2011; Heyer *et al.*, 2012), ChrSA was shown to be indispensable for the detoxification of haem by activating the expression of *hrtBA*, encoding a putative haem export system (Stauff and Skaar, 2009; Heyer *et al.*, 2012). This is in contrast to what has been reported for the homologous *C. diphtheriae* systems. In this prominent human pathogen, the ChrSA system is the main activator of the target genes *hmuO* and *hrtBA*, whereas HrrSA contributes about 20% to the haem-dependent activation of *hmuO* (Bibb *et al.*, 2005; 2007).

HrrSA and ChrSA are both responsive to haem

In this study, activity profiling of P_{hmuO} (fused to *eyfp*) revealed that besides haem, the HrrSA pathway also integrates iron availability, whereas control of *hrtBA* expression by the ChrSA system is only dependent on haem and not influenced by iron (Fig. 2). Even in the presence of very low amounts of haem, HrrS seems to be active and mediates expression of *hmuO*. Phosphorylation of ChrA by ChrS and the associated activation of *hrtBA* expression is initiated at higher haem concentrations and is required to avoid high intracellular haem levels by activation of the haem export system HrtBA. In contrast to the *C. diphtheriae* network, *hrrA* expression is repressed by the global iron-dependent regulator DtxR in *C. glutamicum* when sufficient iron is available (Wennerhold and Bott, 2006). This overall network design allows *C. glutamicum* to preferentially use external iron sources for biosynthesis and to avoid the degradation of *de novo* synthesized haem by the haem oxygenase.

HrrA and ChrA are phosphorylated by HrrS and ChrS

Following the classical model, the response regulators HrrA and ChrA are activated via the phosphorylation of the conserved aspartate residue D54 (Stock *et al.*, 2000; Gao *et al.*, 2007). The mutant strain Δ*hrrS*/Δ*chrS*, lacking both sensor kinases, displayed a similar phenotype with respect to growth and reporter activity (P_{hrtBA} and P_{hmuO}) as the mutant strains lacking the cognate response regulators (Δ*hrrA* or Δ*chrA*) or the mutants where the conserved aspartate residue of HrrA or ChrA has been exchanged to alanine. These results indicate that HrrA and ChrA are activated by the sensor kinases HrrS and ChrS and that likely no other histidine kinase feeds into the HrrSA and ChrSA pathways. Furthermore, they appear not to be phosphorylated by small molecule phosphate donors, as it was described for some response regulators, e.g. for PhoB from *E. coli*, which activates the Pho regulon in the absence of its cognate histidine kinases (Lee *et al.*, 1990; Hiratsu *et al.*, 1995; Wolfe, 2010).

Cross-talk

In their recent review, Podgornaia and Laub highlighted three mechanisms which bacteria engage to ensure specificity of two-component signal transduction; these are (i) molecular recognition, (ii) phosphatase activity and (iii) substrate competition (Podgornaia and Laub, 2013). The first mechanism, molecular recognition, is based on the intrinsic ability of a particular kinase to specifically interact with its cognate regulator. Whereas molecular recognition seems to be an important mechanism to insulate the closely related HrrSA and ChrSA systems from cross-talk with the other TCS in *C. glutamicum*, it did not prevent cross-talk between them. Single deletion mutants of the histidine kinase genes did not display a growth phenotype in the presence of haem and signals of target gene reporters were only slightly altered in comparison to the wild type. These results emphasize that cross-talk between the systems is sufficient to complement mutation of the cognate kinase *in vivo*.

In vitro phosphotransfer studies confirmed cross-talk between the HrrSA and the ChrSA systems, whereas no phosphotransfer to HrrA or ChrA was observed from the PhoS sensor kinase. However, *in vitro* assays suggest that both sensor kinases exhibit a kinetic preference for their cognate response regulator (Fig. 5). Cross-talk, has been described for a number of different examples including the NarX–NarL and NarQ–NarP system of *E. coli* (Stewart, 2003; Noriega *et al.*, 2010), PhoRP and YycGF of *Bacillus subtilis* (Howell *et al.*, 2003; 2006) or the prominent example of CpxA–CpxR and EnvZ–OmpR (Siryaporn and Goulian, 2008; Skerker *et al.*, 2008). However, in most cases, cross-talk seems to be solely a result of genetic perturbations (Podgornaia and Laub, 2013). Our studies revealed intensive cross-talk between HrrSA and ChrSA. In the absence of a stimulus, the bifunctional nature of the sensor kinases (discussed below) is therefore crucial to maintain the OFF state of the particular system.

ChrS and HrrS are bifunctional

Increasing evidence is provided by several studies, demonstrating that most histidine kinases are bifunctional and that phosphatase activity is required to prevent detrimental cross-talk from small-molecule phosphate donors or other kinases (Igo *et al.*, 1989; Laub and Goulian, 2007; Willett and Kirby, 2012; Podgornaia and Laub, 2013). Recent examples include the LiaFSR systems controlling

envelope stress in *B. subtilis* (Schrecke *et al.*, 2013), the vancomycin responsive VanRS system of *Streptomyces coelicolor* (Hutchings *et al.*, 2006), CpxAR and EnvZ–OmpR from *E. coli* (Park and Forst, 2006; Wolfe *et al.*, 2008), or the control of the anti-anti-sigma factor PhyR by the PhyP phosphatase controlling the general stress response in Alphaproteobacteria (Kaczmarczyk *et al.*, 2011).

In this study, we provided *in vitro* and *in vivo* evidence that both sensor kinases, ChrS and HrrS (HisKA_3 subfamily), are bifunctional and that phosphatase activity is required to maintain the OFF state in the absence of a stimulus. Our results further suggest that HrrS acts as a kinase even in the absence of an external haem source (Fig. 6) and that the phosphatase activity of ChrS is crucial to shut the system down when haem levels are tolerable.

Prior studies revealed a glutamine residue within a conserved DxxxQ motif to be important for phosphatase activity of NarX and other HisKA_3 subfamily kinases (Huynh *et al.*, 2010). Remarkably, the exchange of the glutamine residue within the putative DxxxQ phosphatase motif, which is also conserved in HrrS and ChrS, led to a significantly increased activation of target gene reporters, whereas non-target gene reporters were not affected. *In vivo* reporter studies as well as *in vitro* phosphorylation assays clearly demonstrated that both kinases are capable to specifically dephosphorylate their cognate response regulator and confirmed the importance of the conserved glutamine residue for phosphatase activity (Fig. 7).

Several studies focused on the identification of residues involved in kinase and/or phosphatase function (Huynh *et al.*, 2010; 2013; Willett and Kirby, 2012; Willett *et al.*, 2013). Recently, Siryaporn *et al.* evolved robust signal transduction from CpxA to OmpR. By combining cross-talk positive mutations in CpxA a phosphatase activity for OmpR emerged (Siryaporn and Goulian, 2008; Siryaporn *et al.*, 2010). In their model, the authors proposed that this change might be the result of simply an increase in the affinity of CpxA/OmpR interaction. In a systems wide profiling approach of NtrC homologues in *Myxococcus xanthus* TCS Willett and Kirby were able to identify residues which were specifically required for kinase activity and others specifically required for phosphatase activity (Willett and Kirby, 2012). Mutation of these residues did, however, not ultimately lead to a lowered binding affinity. With the two homologue systems HrrSA and ChrSA, which are likely the result of a recent gene duplication event, we describe a pair of TCS, which show a high level of cross-talk, respond to an identical stimulus (haem), but exhibit a highly specific phosphatase activity in their native form. Thus, these two systems represent an ideal model to study the involvement of specific residues catalysing dephosphorylation and of interface residues conferring phosphatase specificity.

Conclusion

Figure 8 illustrates our current model of haem-dependent signal transduction of ChrSA and HrrSA in *C. glutamicum*. Our data confirmed that HrrSA and ChrSA harbour distinct functions in haem homeostasis in *C. glutamicum*. On the one hand the ChrSA system is crucial for haem tolerance by activating *hrtBA* expression in dependence of the external haem concentration. On the other hand, the HrrSA system controls the utilization of haem as alternative iron source, mainly by activating expression of the haem oxygenase gene *hmuO* under iron-limiting conditions. Altogether, our data deliver striking evidence that both, HrrS and ChrS, are bifunctional and suggest phosphatase activity and substrate competition as key mechanisms ensuring pathway specificity of the HrrSA and ChrSA systems. If cross-talk between the two homologous TCS occurs *in vivo*, the phosphatase activity counteracts this by dephosphorylation of its cognate response regulator.

Experimental procedures

Bacterial strains and growth conditions

Corynebacterium glutamicum ATCC 13032 was used as wild type strain (Kalinowski, 2005). For cloning purposes *E. coli* DH5α was used; for overproduction of proteins *E. coli* BL21 (DE3) (Studier and Moffatt, 1986). *E. coli* was cultivated in Lysogeny Broth (LB) medium at 37°C or on LB agar plates. When necessary, 50 μg ml^{-1} kanamycin was added. The bacterial strains used in this study are listed in Table 1.

For reporter assays and growth experiments, cells were inoculated from a BHI pre-culture (brain heart infusion, Difco™ BHI, BD, Heidelberg, Germany) and cultivated in CGXII minimal medium or on CGXII agar plates (Keilhauer *et al.*, 1993) respectively. The media contained glucose as a carbon and energy source and either $FeSO_4$ and/or haemin (protoporphyrin IX with Fe^{3+}) as an iron source. To improve iron uptake, the medium was supplemented with 195 μM protocatechuic acid (PCA), which serves as an Fe^{3+} chelator. When no PCA was added to the growth medium, the cells suffered from severe iron limitation. Cultivation of *C. glutamicum* +/– PCA was performed to study the dose-dependent effect of haem on gene expression in the presence or absence of bioavailable iron.

Phenotyping on agar plates

For growth experiments of *C. glutamicum* ATCC 13032 and deletion mutants on agar plates, the strains were grown in a 5 ml BHIS culture overnight. The cells were diluted in 0.9% (w/v) NaCl to an OD_{600} of 1 and dilution series (3 μl each, 10^0 to 10^{-5}) were spotted on CGXII agar plates containing 4%

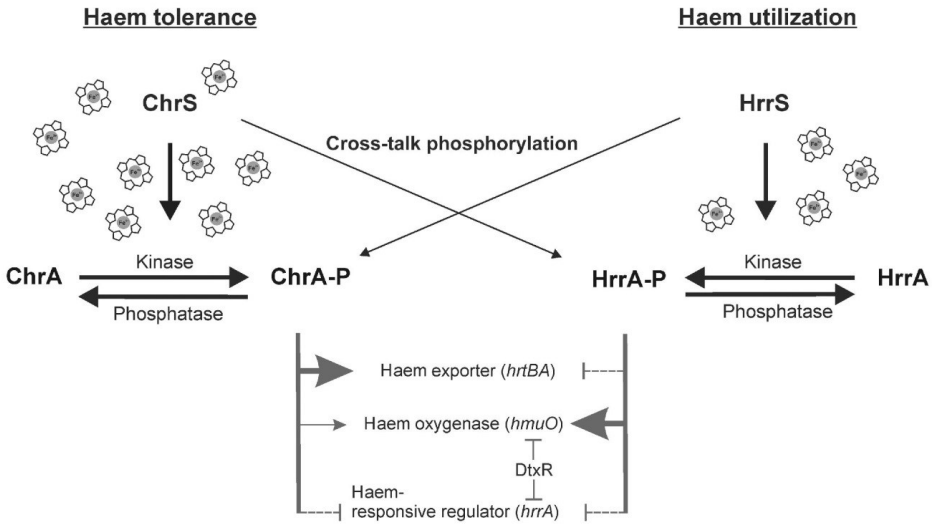

Fig. 8. The current model of HrrSA and ChrSA interaction in *C. glutamicum*. The ChrSA TCS predominantly mediates detoxification of haem by upregulation of *hrtBA* upon high haem levels. The HrrSA TCS is required for utilization of haem as an alternative source of iron by activating *hmuO*. Activation of the response regulators ChrA and HrrA is mediated by their cognate kinases ChrS and HrrS, but a cross-regulation between non-cognate pairs was shown in this study. The kinases HrrS and ChrS have a dual function both as kinase and as phosphatase. Phosphatase activity of each kinase was shown to be specific towards its cognate response regulator, thereby ensuring pathway specificity of these closely related systems. When sufficient iron is available, the genes *hrrA* and *hmuO* are both repressed by regulator of iron homeostasis DtxR.

(w/v) glucose and either 2.5 μM $FeSO_4$ (+PCA) and/or 2.5 μM haemin. When haemin was the only iron source no PCA was added. Pictures were taken after incubation for 48 h at 30°C. When necessary, 25 μg ml^{-1} kanamycin was added.

Cloning techniques

Routine methods were performed according to standard protocols (Sambrook *et al.*, 2001). Chromosomal DNA of *C. glutamicum* ATCC 13032 was prepared (Eikmanns *et al.*, 1994) and utilized as a template for PCR. DNA sequencing and oligonucleotides synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Tables S1 and S2 respectively.

In-frame deletion mutants of *hrrSA* (cg3248, cg3247), *chrSA* (cg2201, cg2200), *hrrS* (cg3248), *hrrA* (cg3247), *chrS* (cg2201), *chrA* (cg2200), *hrrS*/*chrS* (cg3248, cg2201) and *chrS*/*hrrA* (cg2201, cg3247) were constructed via the two-step homologous recombination method as described before (Niebisch and Bott, 2001). Therefore, the corresponding upstream region covering the first 30 bp of e.g. *hrrS* was amplified with primers DhrrS-1 and DhrrS-2 and in a second step the downstream region with the last 30 bp of *hrrS* was amplified with primers DhrrS-3 and DhrrS-4 by PCR. Subsequently, the up- and downstream flanking regions of respective genes were fused via an overlap of 21 bp by overlap extension PCR with primers DhrrS-1 and DhrrS-4 (oligonucleotides are listed in Table S2). PCR products were ligated into pK19*mobsacB* at restriction sites as indicated. The pK19*mobsacB* inserts for other deletion mutants were created analogously. The resulting plasmids pK19*mobsacB*-Δ*chrSA*, pK19*mobsacB*-Δ*hrrSA*, pK19*mobsacB*-Δ*hrrA*, pK19*mobsacB*-Δ*hrrS*, pK19*mobsacB*-Δ*chrS*, and pK19*mobsacB*-Δ*chrA* (plasmids listed in Table S1) were used for the deletion of the corresponding genes in *C. glutamicum* by homologous recombination as described previously (Schäfer *et al.*, 1994). Successful deletion of e.g. *hrrS* was verified by colony PCR using the primers DhrrS-fw and DhrrS-rv and DNA sequencing (oligonucleotides are indicated in Table S2).

The *C. glutamicum* wild type and the deletion strains Δ*hrrSA* and Δ*chrSA* were transformed with the resulting plasmids according to a standard protocol (van der Rest *et al.*, 1999). For complementation of the phenotype of the Δ*hrrSA* mutant strain, DNA fragments covering *hrrSA* and the 119 bp upstream region were amplified. The DNA fragment of *hrrSA* and its native promoter was cloned into the vector pJC1 using the NheI restriction site (Cremer *et al.*, 1990). The open reading frame of *hmuO* (cg2445) with an artificial ribosome binding site (underlined) and an 8 bp spacer in front of the start ATG (AAGGAGATATAGAT) was cloned into the pEKEx2

vector using the restriction sites BamHI and EcoRI under the control of the IPTG-inducible promoter P_{tac}.

For overproduction and purification of the kinase domain of ChrS for *in vitro* phosphorylation studies, the *chrS* region coding for kinase domain was amplified with the oligonucleotides chrS-K-fw and chrS-K-rv (Table S2). Subsequently, the DNA fragment was cloned into the expression vector pMal-c via PstI and HindIII restriction sites, resulting in the plasmid pMBP-ChrSΔ1–176, which enabled the overexpression of the ChrS kinase domain fused to the C-terminus of the *E. coli* maltose-binding protein (MBP) lacking its signal peptide.

For mutagenesis of the conserved DxxxQ phosphatase motif (ChrS-Q191A and HrrS-Q222A) within pMBP-ChrSΔ1–176 and pMBP-HrrSΔ1–169, amino acid exchanges were generated by overlap PCR. Exemplified for pMBP-ChrS-Q191AΔ1–176, the corresponding upstream region of *chrS* was amplified by PCR via primers chrS-K-fw and chrS-Q191A-2 and the corresponding downstream region with primers ChrS-Q191A-3 and chrS-K-rv, carrying the desired mutation in the ChrS phosphatase domain. Subsequently, the resulting fragments were fused via overlap extension PCR using the primers chrS-K-fw and chrS-K-rv (Table S2). Cloning was performed as described above. Further plasmids were created analogously.

For construction of plasmids for overproduction of the cytoplasmatic kinase domain of PhoS, HrrS and the response regulators PhoR, HrrA and ChrA see references (Schaaf and Bott, 2007; Frunzke *et al.*, 2011; Heyer *et al.*, 2012). The construction of promoter fusions of the promoters of *hrtBA* and *hmuO* with *eyfp* was described previously (Heyer *et al.*, 2012).

Site-directed mutagenesis

For mutagenesis of the conserved DxxxQ phosphatase motif (HrrS-Q222A and ChrS-Q191A) and mutagenesis of the conserved phosphorylated aspartate residues (HrrA-D54A and ChrA-D54A) amino acid exchanges were generated via site-directed mutagenesis. Therefore, exemplified here for HrrA-D54A, the corresponding upstream region of *hrrA* was amplified by PCR via primers hrrA-D54A-1 and hrrA-D54A-2 and the corresponding downstream region with primers hrrA-D54A-3 and hrrA-D54A-4, carrying the desired mutation in the HrrA phosphorylation domain. Subsequently, the resulting fragments were fused via overlap extension PCR using the primers hrrA-D54A-1 and hrrA-D54A-4 (Table S2). PCR products were finally ligated into pK19*mobsacB* with EcoRI and BamHI restriction sites. Further plasmids were created analogously. The resulting plasmids pK19*mobsacB*-HrrS-Q222A and pK19*mobsacB*-ChrS-Q191A were used for the genomic exchange of glutamine to alanine at position 222 for HrrS and 191 for ChrS and the plasmids pK19*mobsacB*-HrrA-D54A and pK19*mobsacB*-ChrA-D54A, were used for the exchange of aspartate to alanine at position 54 in the respective *C. glutamicum* strains by homologous recombination as described previously (Schäfer *et al.*, 1994).

Reporter assays

For reporter assays a 20 ml pre-culture of CGXII minimal medium containing 2% (w/v) glucose was inoculated from a 5 ml BHI culture after washing the cells with 0.9% (w/v) NaCl. To adjust *C. glutamicum* to iron starvation conditions, no iron source (haem or $FeSO_4$), but PCA was added to the second pre-culture, allowing the uptake of trace amounts of iron present in the growth medium. Cells were incubated overnight at 30°C and 120 r.p.m. in a rotary shaker and grew to an OD_{600} of ~ 20. For all reporter assay experiments, PCA was added to the CGXII minimal medium. Reporter assays in microtitre scale were performed in the BioLector system (m2p-labs GmbH, Aachen, Germany). Therefore, 750 µl CGXII medium containing 2% (w/v) glucose and different concentrations of $FeSO_4$ and/or haemin were inoculated from the second pre-culture with iron-starved cells (0 µM $FeSO_4$ + PCA) to an OD_{600} of 1 and cultivated in 48-well Flowerplates® (m2p-labs GmbH, Aachen, Germany) at 30°C, 95% humidity, 1200 r.p.m. (900 r.p.m. for activity profiling) and a shaking diameter of 3 mm. The $FeSO_4$ or haemin solutions were added from a sterile filtered stock solution after autoclaving, as indicated. For the haemin stock solution, haemin (Sigma Aldrich, Munich, Germany) was dissolved in 20 mM NaOH to a concentration of 250 µM. When necessary, 25 µg ml^{-1} kanamycin was added.

For activity profiling, concentrations of 0–36 µM $FeSO_4$ and 0–8 µM haemin in all possible combinations were applied. $FeSO_4$ was added in combination with PCA to ensure rapid iron uptake. To adjust severe iron limitation, cells were additionally cultivated in the absence of both $FeSO_4$ and PCA. The production of biomass was determined as the backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor of 12); measurements were taken in 10 min intervals. For promoter fusion studies the eYFP chromophore was excited at 510 nm and emission was measured at 532 nm (signal gain factor of 50). The specific fluorescence (arbitrary units, a.u.) was calculated as the ratio of the eYFP fluorescence signal and the backscatter signal (Kensy *et al.*, 2009). To correct for background fluorescence e.g. of the media, fluorescence of respective strains carrying the empty vector as negative control was subtracted from all measurements.

Overproduction and purification of histidine kinases and response regulators

Overproduction of PhoR, PhoS (Schaaf and Bott, 2007), HrrA, HrrS (Frunzke *et al.*, 2011) and ChrA (Heyer *et al.*, 2012) was performed as described before. For overproduction of ChrS, ChrS-Q191A and HrrS-Q222A *E. coli* BL21(DE3) was transformed with the vector pMBP-ChrSΔ1–176, pMBP-ChrS-Q191AΔ1–176 or pMBP-HrrS-Q222AΔ1–169 and cultivated in 200 ml LB medium at 37°C and 100 r.p.m. At an OD_{600} of ~ 0.7, expression was induced by addition of 1 mM IPTG. After 4 h at 30°C cells were harvested by centrifugation (4000 *g* at 4°C, 10 min). The cell pellet was stored at –20°C. For protein purification, the cell pellet was resuspended in TNM buffer (50 mM Tris-HCl, pH 8.0, 200 mM NaCl, 5 mM $MgCl_2$), containing Complete protease inhibitor cocktail (Roche, Germany). Cells were disrupted by passing a French pressure cell (SLM Ainco, Spectronic Instruments, Rochester, NY) two times at 207 MPa. The cell

debris was removed by centrifugation (6900 *g*, 4°C, 20 min), followed by an ultracentrifugation of the cell-free extract for 1 h (150 000 *g*, 4°C). MBP proteins present in the supernatant after ultracentrifugation were purified by affinity chromatography on amylose resin (New England BioLabs). Equilibration was performed with TNM buffer. After washing with 15 column volumes of TNM buffer, MBP proteins were eluted with three column volumes of TNM buffer containing 10 mM maltose. Fractions containing the desired MBP protein were pooled, the buffer was exchanged against phosphorylation buffer [20 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$, 5% (v/v) glycerol, 0.5 mM EDTA, 0.005% (w/v) Triton X-100] using a PD10 desalting column (GE Healthcare, Munich, Germany). The purified MBP protein was kept at 4°C and was used immediately for phosphorylation studies. Purification of proteins was analysed on a 12% SDS-polyacrylamide gel and staining of the MBP proteins was performed with Coomassie brilliant blue. The protein concentration was determined with Bradford reagent (Bradford, 1976) using bovine serum albumin as standard. After storage for about 2 days at 4°C, kinase activity was significantly reduced.

In vitro *phosphorylation assays*

To determine the autophosphorylation activity of MBP-ChrSΔ1–176, 12 μM of MBP-ChrSΔ1–176 were incubated with 0.25 μM [γ-^{33}P]-ATP (10 mCi ml^{-1}; Hartmann Analytic GmbH Germany) mixed with 80 μM non-radioactive ATP. The assay mixture (75 μl) was incubated at room temperature and at different times, 7 μl aliquots were removed, mixed with an equal volume of 2× SDS loading buffer [124 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4.6% (w/v) SDS, 1.4 M β-mercaptoethanol, 0.01% (w/v) bromophenol blue] and kept one ice. Without prior heating, the samples were subjected to SDS-PAGE (12% separating gel). After being dried, the gel was analysed with a Typhoon Trio Scanner (GE Healthcare, Germany). For analysis of cross-talk from the histidine kinases MBP-HrrSΔ1–169, MBP-HrrS-Q222AΔ1–169, MBP-ChrSΔ1–176, MBP-ChrS-Q191AΔ1–176 and MBP-PhoSΔ1–246 to the response regulators HrrA, ChrA, and PhoR, a twofold molar excess of purified response regulators (12 μM) was added to the respective histidine kinases (diluted to 6 μM) in the assay mixture described above and the samples were incubated at room temperature. At different time points, aliquots were taken and further processed as described above.

Sequence analysis

Amino acid sequence alignments were performed using the online tools NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/). For prediction of the membrane topology of ChrS and HrrS N-terminal regions the online transmembrane prediction programs TopPredII (http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) and HMMTOP (http://www.sacs.ucsf.edu/cgi-bin/hmmtop.py) were used. Prediction of domains was performed with the help of Pfam database (http://pfam.sanger.ac.uk/).

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Conflict of interest

The authors declare that they have no competing interests.

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Identification of residues involved in phosphatase activity of the two-component systems HrrSA and ChrSA in *Corynebacterium glutamicum*

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Summary

Two-component systems (TCS) are the prevailing tool for bacteria to transform environmental stimuli into an adequate response of their gene expression pattern. A single bacterial genome can contain up to dozens of highly related TCS. Dictating specificity in the concert of signal-transduction is a major challenge for bacteria. The Gram-positive soil bacterium *Corynebacterium glutamicum* expends two homologous TCS named HrrSA and ChrSA for the control of haem homeostasis. The phosphatase activity of the kinases HrrS and ChrS, which is specific only for their cognate response regulators, is a crucial feature to ensure pathway specificity in these cross-talking systems. However a putative conserved phosphatase motif (DxxxQ) is identical for both systems. In this study we established an error prone library of HrrS and screened for further putative catalytical residues involved in phosphatase reaction. Using the target gene reporter P_{hmuO}-*venus* enabled a single cell screening analysis of error prone libraries and identification of residues involved in phosphatase activity which are highly conserved among haem-dependent TCS. Besides phosphatase activity, pathway specificity can further be enhanced by molecular recognition. Analysis of chimeric proteins of HrrS and ChrS delivered first evidence, that residues forming the interface during phosphatase reaction are located inside the dimerization and histidine phosphotransfer domain. The present data shed light on the residues involved in phosphatase activity of these highly intertwined haem-dependent TCS HrrSA and ChrSA in *C. glutamicum*.

Introduction

Two component-systems (TCS) enable bacteria to transform the high diversity of extracellular stimuli into an adequate adjustment of their gene expression pattern and cellular physiology (Mascher *et al.*, 2006, Stock *et al.*, 2000). The composition of a typical TCS includes a membrane bound histidine-kinase (HK) and its cognate response regulator (RR). Signal recognition, mediated by the HK leads to an autophosphorylation of the conserved histidine residue, located inside the dimerization and histidine phosphotransfer (DHp) domain. The signal is further transduced by the transfer of this phosphoryl group to an invariant aspartate residue residing in the response regulator receiver domain (REC), which then leads to the adaptation of cellular gene expression (Stock *et al.*, 2000).

Considering the enormous number of TCS existing in some bacterial genomes, there have to be forces ensuring that a HK can discriminate its cognate RR to maintain the correct flux of information. Typically, the number of bacterial TCS increases with the genome size and the environmental complexity (Alm *et al.*, 2006). It was shown, that bacteria have enlarged their TCS repertoire by gene duplication events and lateral gene-transfer during the course of evolution (Alm *et al.*, 2006). As HKs and RRs both involve paralogous gene families, sharing often a high sequence similarity there is a high

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potential of cross-talk between non-cognate partners (Capra and Laub, 2012, Galperin, 2005). Three mechanisms were described ensuring phosphotransfer specificity in TCS signal transduction: (i) Substrate competition, (ii) phosphatase activity of the sensor kinase, and (iii) insulation *via* changes in specificity residues (Podgornaia and Laub, 2013). The most important mechanism to ensure insulation of signal transduction pathways is molecular recognition. This is based on the assumption that a HK has the intrinsic ability to discriminate its cognate RR from the crowded milieu of non-cognate RRs. A relatively small subset of residues in the interface between HKs and RRs is responsible for maintaining this specificity. These residues were shown to be located in a specific α-helix of the HK and the cognate RR (Skerker *et al.*, 2008, Laub and Goulian, 2007, Capra *et al.*, 2012). As a further mechanism, substrate competition depends on the stoichiometry of HK and RR. In most cases the RR outvalues the level of HK and thereby outcompetes non-cognate partners as described for the EnvZ-OmpR TCS from *E. coli* (Groban *et al.*, 2009, Siryaporn and Goulian, 2008). Finally, phosphatase activity denotes the bifunctional nature of HKs, which often catalyze not only phosphorylation, but also dephosphorylation of their cognate RRs. This ensures the elimination of an inappropriate RR phosphorylation by non-cognate HKs or small phospho-donors like acetyl phosphate (Huynh and Stewart, 2011, Igo *et al.*, 1989).

In the Gram-positive soil bacterium *Corynebacterium glutamicum*, two TCS named HrrSA and ChrSA play the key roles in the control of haem homeostasis (Frunzke *et al.*, 2011, Heyer *et al.*, 2012). These highly intertwined TCS, share a high sequence similarity with each other as well as with the orthologous TCS from the close relative *Corynebacterium diphtheriae* (Hentschel *et al.*, 2014, Bibb *et al.*, 2007, Bibb and Schmitt, 2010). The function of both systems was characterized recently in *C. glutamicum*. The TCS HrrSA is important for the utilization of haem as an alternative source of iron, as it is the main activator of the expression of *hmuO* encoding the haem-oxygenase. Furthermore HrrSA regulates the expression of genes encoding for respiratory chain components and for haem biosynthesis enzymes (Frunzke *et al.*, 2011). To allow the preferential utilization of iron, *hrrA* and *hmuO* expression both underlie the repression of DtxR, the master regulator of iron homeostasis (Wennerhold and Bott, 2006). In contrast to that, the homologous TCS ChrSA is required to mediate resistance against high levels of haem by the haem-dependent activation of the expression of *hrtBA* encoding for the putative haem exporter HrtBA (Heyer *et al.*, 2012).

Previous work uncovered that the TCS HrrSA and ChrSA exhibit a high level of cross-talk and pointed out phosphatase activitiy of the HKs as a crucial feature for ensuring specificity in signal transduction (Hentschel *et al.*, 2014). This phosphatase activity of HrrS and ChrS was shown to be highly specific only for the cognate RR. For both HrrS and ChrS a conserved glutamine residue was identified to be important for the catalyzation of this phosphatase activity. Remarkably, although phosphatase activity seems to be highly specific, this glutamine residue is part of a completely identical phosphatase motif (DTVAQ), inside the DHp domain of HrrS and ChrS (Hentschel *et al.*, 2014).

Thus this study aims at the identification of further catalytical residues involved in the phosphatase activity of the TCS HrrSA and ChrSA. Sequence analysis of the DHp domains of haem-dependent TCS of different *Corynebacterial* species shed light on the conserved phosphatase motif and the DHp domain architecture. Furthermore an alignment of the different HisKA and HisKA_3 type kinases of *C. glutamicum* identified different putative phosphatase motifs for both subclasses and suggests phosphatase activity as a general mechanism for all 13 TCS in *C. glutamicum*.

Additionally, construction and screening of an error prone library of the HrrS DHp domain using the target gene reporter P_{hmuO}-*venus*, revealed first hints for further catalytical residues involved in phosphatase activity besides the conserved glutamine residue. For the localization of the protein interface conferring specificity during phosphatase reaction, chimeric proteins were constructed and analysed *via* target gene reporters (P_{hmuO}-*eyfp* and P_{hrtBA}-*eyfp*). This work study provides first hints that the subset of interface residues dictating phosphatase specificity is located in the DHp domains of HrrS and ChrS.

Table 1. Bacterial strains used in this study.

Strains	Characteristics	Reference
Escherichia coli		
E. coli DH5α	*supE44* Δ*lacU169* (ϕ80*lacZ*DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
C. glutamicum		
ATCC13032	Biotin-auxotrophic wildtype	(Kinoshita *et al.*, 2004)
Deletion mutants	**In-frame deletion of operons/genes**	
ATCC13032 Δ*hrrS*/ Δ*chrS*-cg1121/22::P_{hmuO}-*venus*	Mutants lacking *hrrS* (cg3248) and *chrS* (cg2200) containing a genomically integrated P_{hmuO}-*venus* reporter (integrated in intergenic region between cg1121/22)	This work
Deletion mutants	**Mutation of phosphatase domain: amino acid exchange of glutamine to alanine**	
ATCC13032 HrrS-Q222A	Amino acid exchange of catalytic residue in the phosphatase domain (glutamine to alanine) in *hrrS* (cg3248) at position 222	(Hentschel *et al.*, 2014)
ATCC13032 ChrS-Q191A	Amino acid exchange of catalytic residue in the phosphatase domain (glutamine to alanine) in *chrS* (cg2200) at position 191	(Hentschel *et al.*, 2014)
Chimeras	**Exchanges of the HrrS and ChrS DHp domains in Phosphatase OFF strains**	
ATCC13032 HrrS-Q222A, HrrS Ex1	Exchange of the complete DHp domain of ChrS 177-242 (cg2200) against HrrS 208-281 (cg3248)	This work
ATCC13032 HrrS-Q222A, HrrS Ex2	Exchange of the partial DHp domain of ChrS 202-242 (cg2200) against HrrS 233-281 (cg3248)	This work
ATCC13032 HrrS-Q222A, HrrS Ex3	Exchange of the partial DHp domain of ChrS 202-216 (cg2200) against HrrS 233-255 (cg3248)	This work
ATCC13032 ChrS-Q191A, ChrS Ex1	Exchange of the complete DHp domain of HrrS 208-281 (cg3248) against ChrS 177-242 (cg2200)	This work
ATCC13032 ChrS-Q191A, ChrS Ex2	Exchange of the partial DHp domain of HrrS 233-281 (cg3248) against ChrS 202-242 (cg2200)	This work
ATCC13032 ChrS-Q191A, ChrS Ex3	Exchange of the partial DHp domain of HrrS 233-255 (cg3248) against ChrS 202-216 (cg2200)	This work

Results

Phylogenetic relationship and conservation of *C. glutamicum* kinases

The genome of *C. glutamicum* encodes 13 TCS, which are all composed of the prototypical type consisting of a HK and its cognate RR (Bott and Brocker, 2012). Up to now, besides the haem-dependent TCS HrrSA and ChrSA, the function of only a few TCS was described more in detail (Bott and Brocker, 2012, Hentschel *et al.*, 2014). Seven of the *C. glutamicum* HKs belong to Histidine Protein Kinase subfamily HPK1, one to HPK5, and the remaining five to HPK7 (Grebe and Stock, 1999, Bott and Brocker, 2012). According to Pfam and based on their DHp domain architecture, the 13 HKs represent two subgroups, the HisKA (CgtS1, CgtS2, CgtS4, CgtS5, MtrB, PhoS, and CopS) and the HisKA_3 type kinases (HrrS, ChrS, CgtS6, CgtS7, and CgtS10) (Bott and Brocker, 2012, Finn *et al.*, 2014). Solely CitA can not be assigned in any of these groups. The HisKA subfamily (pfam00512), with 77% found within 1500 sequenced microbial genomes includes the majority of sequences, whereas the HisKA_3 subfamily (pfam07730), comprises about 10% of DHp domain sequences (Finn *et al.*, 2014) and corresponds to the HPK7 transmitter subfamily of Grebe and Stock (Grebe and Stock, 1999). A phylogenetic analysis with protein sequences of the 13 kinases of *C. glutamicum* revealed, that both HisKA and HisKA_3 type groups form clusters (Fig. 1A). The split into HisKA and HisKA_3 type kinases presumably occured in the

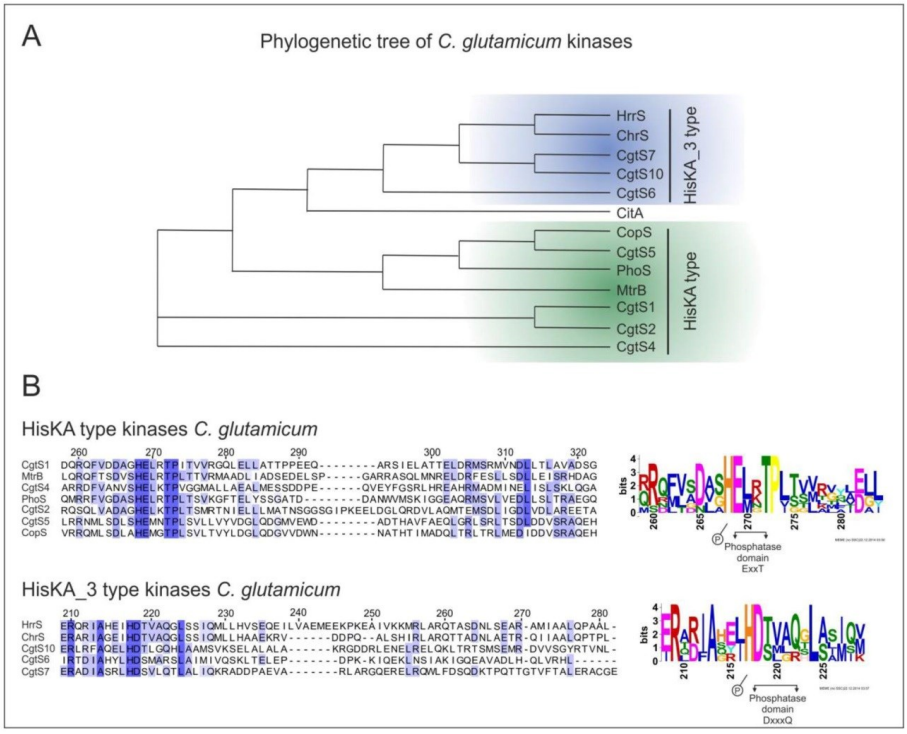

Figure 1: Phylogenetic analysis and DHp conservation of *C. glutamicum* ATCC13032 kinases. A. For the phylogenetic analysis, protein sequences of *C. glutamicum* kinases were obtained from CoryneRegnet. Classification of HisKA and HisKA_3 type kinases was performed using the Pfam database. Phylogenetic analysis was performed using ClustalW online tool. **B**. Alignments of the DHp domains of the *C. glutamicum* HisKA and HisKA_3 type kinases was performed using Clustal W, motif search was performed with the help of the MEME sutite. Numbering according to CgtS1 and HrrS.

earlier steps of evolution. Several gene duplication events might have led to this diversity of TCS. Maintaining the correct flux of signal transduction after these TCS gene duplication events requires an insulation *via* changes in interface residues (Podgornaia and Laub, 2013). According to the phylogenetic tree the haem dependent HKs HrrS and ChrS are closely related (HrrS and ChrS: 39%; HrrA and ChrA: 57% identity) and share a common ancestor with the also highly related TCS CgtS7 and CgtS10 (Fig. 1A). In prior studies it was shown, that phosphatase activity of these cross-talking and highly related TCS HrrSA and ChrSA, is a determinant of specificity (Hentschel *et al.*, 2014). Protein sequence comparison and motif analysis of the two different HisKA and HisKA_3 subfamily HKs in *C. glutamicum* revealed two different conserved phosphatase motifs in close proximity to the conserved histidine residue for the two groups (Fig. 1B). The motif ExxT is highly conserved for HisKA type kinases and a DxxxQ motif (except for CgtS6 - DxxxR) is highly conserved for HisKA_3 type kinases. This is consistent with prior findings, demonstrating the importance of such motifs for catalytical phosphatase activity (Huynh *et al.*, 2010, Willett and Kirby, 2012).

Conservation of DHp and REC domains of HrrS and ChrS

The control of the haem homeostasis by the two TCS HrrSA and/or ChrSA is widespread among *Corynebacterial* species (Hentschel *et al.*, 2014, Bibb *et al.*, 2007). The HKs ChrS and HrrS typically sense the presence of haem to adapt the cellular physiology to the certain environmental conditions *via* response regulator phosphorylation and dephosphorylation. To identify highly conserved putative phosphatase residues in haem dependent kinases, an alignment of HrrS and ChrS from different *Corynebacterial* species was performed (Fig. 2A). A high density of conserved residues can be found in the first α-helix (according to HrrS) of the DHp domain. Immediately adjacent to the invariant His-10 residue, the highly conserved DTxAQ motif can be found, which was recently demonstrated to be crucial for phosphatase activity of HrrS and ChrS in *C. glutamicum* (Hentschel *et al.*, 2014). This motif, also highlighted in the weblogo is surrounded by further conserved putative catalytical amino acids forming the H-box (Fig. 2A). Only few conserved residues can be found in the N-terminal region of the second α-helix (LARxTAADNL).

In *C. glutamicum* both TCS HrrSA and ChrSA, which share a high sequence similarity are present. An alignment of DHp domains of HrrS and ChrS from *C. glutamicum* highlights the high sequence similarity especially in the first α-helix (Fig. 2B). These highly conserverd residues might be further putative candidates for catalytical phosphatase activity besides the conserved glutamine residue inside the phosphatase motif (DxxxQ) (Hentschel *et al.*, 2014). Thus, the residues involved in phosphatase reaction seem to be highly conserved, it is feasable that only the interface of HrrS and ChrS changed during the course of evolution. It was shown that these interface residues, being important for the molecular recognition between HK and its cognate RR are located in the first α-helix of the response regulator receiver domain (REC) and in both helices of the DHp domain of the kinase (Podgornaia and Laub, 2013). Figure 2B illustrates that for the TCS HrrSA and ChrSA from *C. glutamicum* both domains DHp and REC share a high sequence similarity with each other. Only few residues in the helices of these domains are not conserved and might play a critical role in forming the interface. Especially in the first α-helix of the HrrA and ChrA REC domain an accumulation of hydrophobic amino acids can be observed. The following experiments aim to identify further catalytical and interface residues, being involved in the phosphatase reaction of the TCS HrrSA and ChrSA in *C. glutamicum*.

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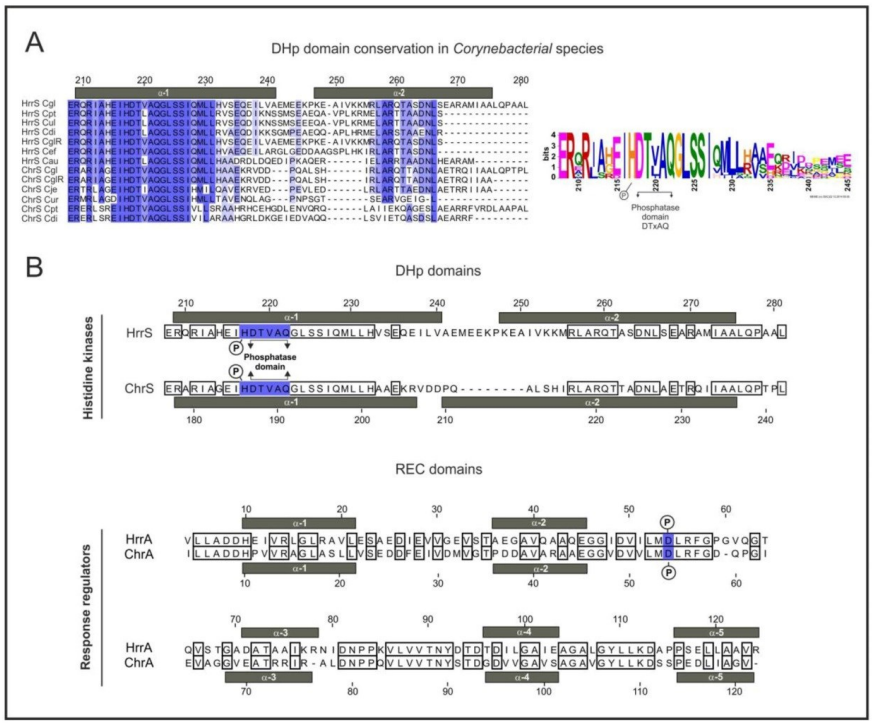

Figure 2: DHp domain conservation of selected *Corynebacterial* species and interface analysis of HrrS/ChrS DHp and HrrA/ChrA REC domains. A. For HrrS and ChrS DHp domain conservation analysis, protein sequences of HrrS and ChrS of selected *Corynebacterial strains* were used: Cgl, *Corynebacterium glutamicum* ATCC 13032 (Kalinowski *et al.*, 2003, Ikeda and Nakagawa, 2003); CglR, *Corynebacterium glutamicum* R (Yukawa *et al.*, 2007); Cau, *Corynebacterium aurimucosum* ATCC 700975 (Trost *et al.*, 2010a); Cdi, *Corynebacterium diphtheriae* NCTC-13129 (Cerdeno-Tarraga *et al.*, 2003); Cef, *Corynebacterium efficiens* YS-314 (Nishio *et al.*, 2003); Cje, *Corynebacterium jeikeium* K411 (Tauch *et al.*, 2005); Cpt, *Corynebacterium pseudotuberculosis* FRC41 (Trost *et al.*, 2010b); Cur, *Corynebacterium urealyticum* DSM 7109 (Tauch *et al.*, 2008); Cul, *Corynebacterium ulcerans* BR-AD22 (Trost *et al.*, 2011). HisKA_3 domain identification was performed using Pfam database. Alignments of the DHp domains was performed using Clustal W, motif search was performed with the MEME suite. Numbering according to HrrS. **B**. For the analysis of the HrrSA/ChrSA interface, domains were predicted using the Pfam database. Alignments were performed using ClustalW and topology prediction of the *C. glutamicum* ATCC13032 HrrS/ChrS DHp and HrrA/ChrA REC domains, was performed using the PSIPRED3 secondary structure prediction program (Jones, 1999).

Identification of further catalytical phosphatases residues

To identify further catalytical residues involved in phosphatase reaction, a mutant library of the HrrS DHp domain was established in *C. glutamicum*. To exclude the formation of heterodimers and the influence of cross-talk the strain Δ*hrrS*/Δ*chrS* was chosen. To enable a screening, the genomically integrated (intergenic region between cg1121/cg1122) target gene reporter P_{hmuO}-*venus* was used. In prior studies it was shown, that kinase ON/Phosphatase OFF mutants show a strongly increased reporter output, which provides a basis for the screening approach chosen for this

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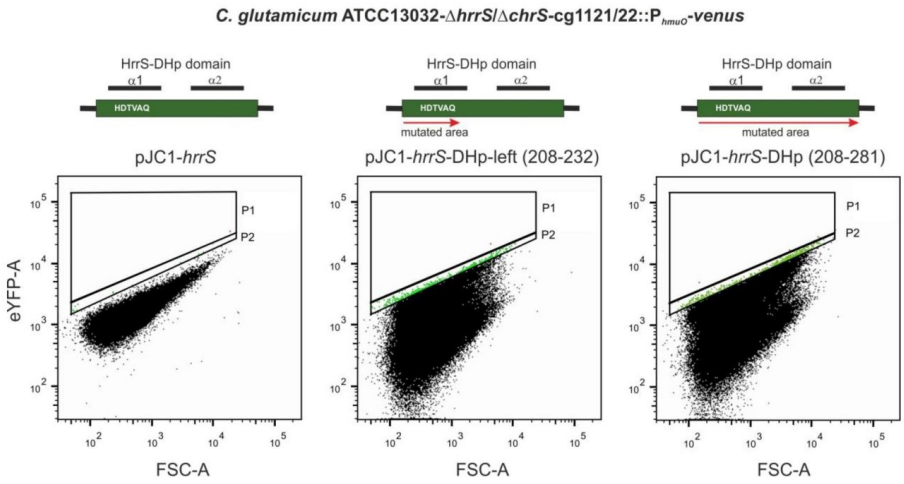

Figure 3: Error prone library screening for single Phosphatase OFF cells. The *C. glutamicum* ATCC13032 strain Δ*hrrS*/Δ*chrS* conatining the genomically integrated target gene reporter P_{hmuO}-*venus* was used for screening of the libraries. The three FACS plots illustrate, from left to right, the control with the wild-type HrrS, the positive control with cells containing the error-prone PCR library of HrrS. The stringent gate P1 and gate P2 with lower fluorescent cells was used for sorting.

study (Hentschel *et al.*, 2014). The P_{hmuO}-*venus* reporter represents a valuable tool for the FACS screening to isolate cells with increased fluorescent signal and phosphatase OFF muteins. The *hrrS* DHp coding sequence (HrrS 208-281) and the left part of the DHp domain (HrrS 208-232), containing the conserved H-Box were amplified using the Diversify PCR Random Mutagenesis Kit (Clontech) to introduce one mutation per sequence (Fig. 3). The resulting library of approximately 10^5 clones was established in the *E. coli* strain Top10. Transformants were pooled, the plasmid DNA was isolated, and the library was transferred into *C. glutamicum* Δ*hrrS*/Δ*chrS* with the integrated target gene reporter P_{hmuO}-*venus*. As negative control the empty vector pJC1 was chosen, which displayed no fluorescent output (data not shown). The wildtype HrrS protein, expressed under the control of its native promoter from pJC1-*hrrS* in Δ*hrrS*/Δ*chrS*-cg1121/cg1122:: P_{hmuO}-*venus* was used to set the FACS gates (Fig. 3). For sorting, the gates P2 and, as a more stringent one, gate P1 were chosen. The majority of higher fluorescent cells fell into gate P2 (3.5% for the HrrS-DHp library and 2.4% for the HrrS-DHp-left library), whereas only 0.03% of higher fluorescent cells fell into the more stringent gate P1 for both libraries (Fig. 3).

About 10^5 cells of the mutant library were analyzed, and 96 positive cells per gate were selected and directly sorted on BHI agar plates and later analyzed by sequencing of the DHp domain. For the analysis of the library, sequencing results with more than one amino acid substitution were excluded. For the more stringent gate P1, for both libraries HrrS-DHp-left (blue bars) and HrrS-DHp (grey bars), 18 different mutations could be identified in total by this random mutagenesis approach (Fig. 4). The majority of amino acid substitution were located in the C-terminal region of the first α-helix of the DHp domain (Q222R, S225T, I227T, Q228R, M229K, H232T/R, E237K, V240D) and in the N-terminal region of the second α-helix (E249G, V252A, K253R, K254R, M255I, R256C, D264V), whereas only one substitution was located in the N-terminal region of the first α-helix (Q210R) and only two in between both α-

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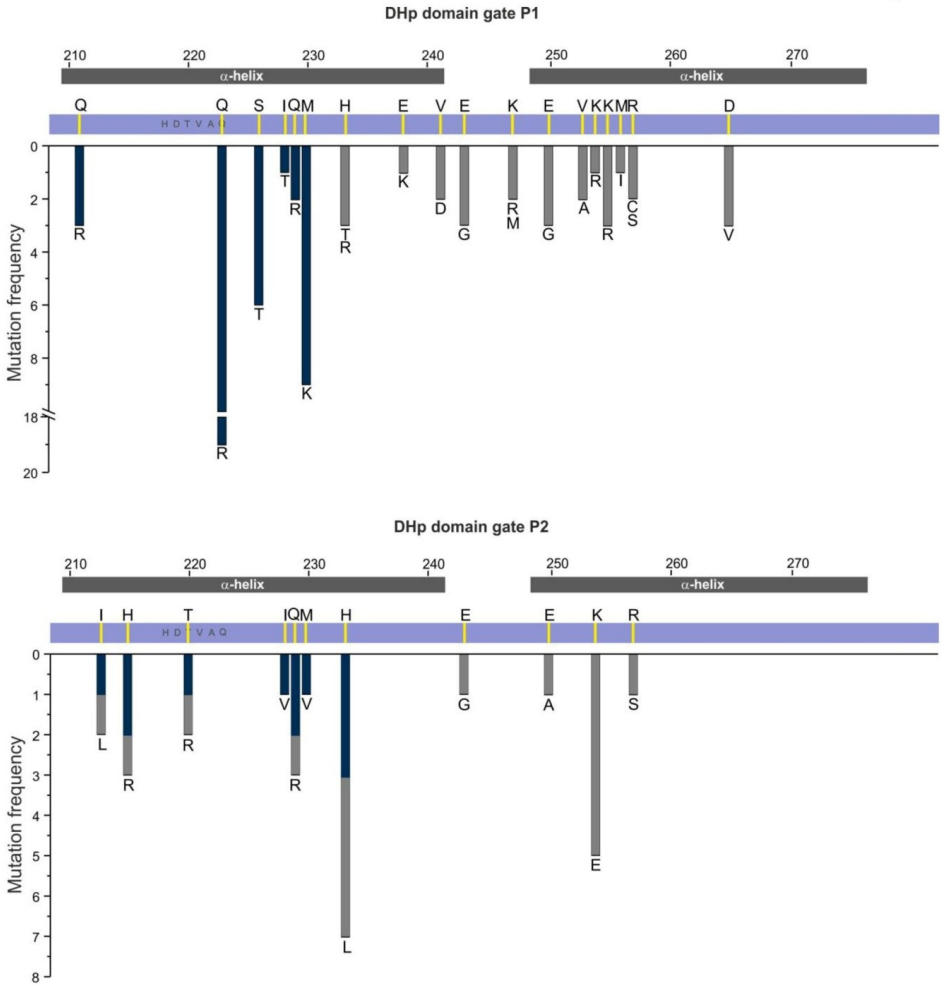

Figure 4: Isolated HrrS muteins Overview of the isolated HrrS DHp domain (208-281) muteins from gate P1 (A) and gate P2 (B). Isolated HrrS muteins and the number of isolated clones of the HrrS-DHp (208-281) and HrrS-DHp-left library (208-232) are indicated as blue and grey bars, repectively. Positions of amino-acid exchanges are indicated in yellow. Predicted α-helix positions are scaled above.

helices (E242G and K246R/M). For the lower gate P2, three further substitutions, located in the N-terminal region of the first α-helix could be identified (I212L, H214R, T219R). The other mutations leading to a higher fluorescent output were identical to those identified for gate P1 (Fig. 4). The highest mutation frequency could be observed for the glutamine residue at position HrrS222. This corresponds to the results of prior studies, which revealed this glutamine residue to be crucial for phosphatase activity of HrrS (Hentschel *et al.*, 2014). Other residues, which were selected with a higher frequency are S225T, M229K/V, H232T/R/L and K253E/R.

Interface analysis of the TCS HrrSA and ChrSA

As the previously described catalytical phosphatase motif (DTVAQ), inside the DHp domain of HrrS and ChrS is identical, we aimed

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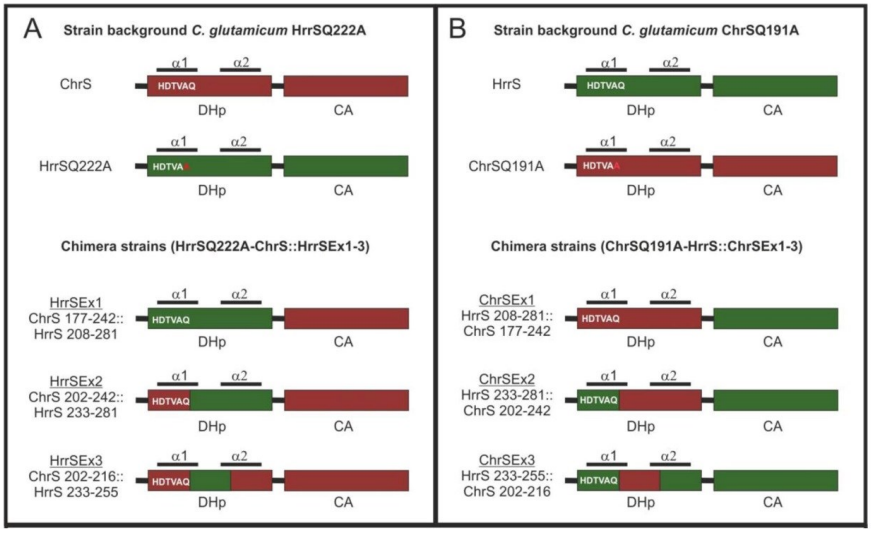

Figure 5: Overview of constructed chimera strains. A: As background strain the phosphatase OFF strain HrrSQ222A was used. In this HrrSQ222A strain background the ChrS DHp domain (red) or parts thereof where exchanged by the corresponding regions of HrrS (green). HrrSEx1: Exchange of the complete ChrS DHp domain by the HrrS DHp domain (ChrS 177-242::HrrS 208-281), HrrSEx2: Exchange of ChrS 202-242::HrrS 233-281, HrrSEx3: Exchange of ChrS 202-216::HrrS 233-255. B: As background strain the phosphatase OFF strain ChrSQ191A was used. In this ChrSQ191A strain background the HrrS DHp domain (green) or parts thereof where exchanged by the corresponding regions of ChrS (red). ChrSEx1: Exchange of the complete HrrS DHp domain by the corresponding ChrS DHp domain (HrrS 208-281::ChrS 177-242), ChrSEx2: Exchange of HrrS 233-281::ChrS 202-242, ChrSEx3: Exchange of HrrS 233-255::ChrS 202-216.

to identify the protein interface conferring phosphatase specificity in ChrSA/HrrSA signaling processes. Some studies of the recent years could show that a small subset of amino acids forming the interface and dictating the interaction specificity between a HK/phosphatase and its cognate RR, are located in the DHp domain of the kinase (Skerker *et al.*, 2008, Podgornaia *et al.*, 2013). The HrrS and ChrS phosphatase OFF phenotypes (HrrSQ222A and ChrSQ191A) were used as strain background for this approach. In this strain background, we constructed chimera kinases by exchanging the whole DHp domains and parts of the non-conserved DHp domain resulting in HrrS/ChrS chimera kinases, to switch phosphatase activity of the respective HK for the non-cognate response regulator (Fig. 5). This approach will be described more in detail exemplified for the construction of the ChrS::HrrSEx1-3 chimeras (Fig. 5A). To switch phosphatase specificity of ChrS from ChrA to HrrA we made use of the phenotype of a HrrS phosphatase OFF/Kinase ON mutein (HrrSQ222A). In this strain background the HrrA target reporter P_{hmuO}-*eyfp* shows a more than 3-fold increased fluorescence in comparison to the wild type strain whereas the reporter output of the P_{hrtBA}-*eyfp* fusion was not affected (Hentschel *et al.*, 2014). By the creation of the chimeras, where the complete ChrS DHp domain (HrrSEx1 – ChrS 177-242::HrrS 208-281) or parts of this domain (HrrSEx2 – ChrS 202-242::HrrS 233-281 and HrrSEx3 – ChrS 202-216::HrrS 233-255) were exchanged by those of HrrS, we aimed to switch phosphatase specificity in a way that finally ChrS is able to dephosphorylate HrrA (Fig. 5A). The chimera strains and as control, the wildtype and

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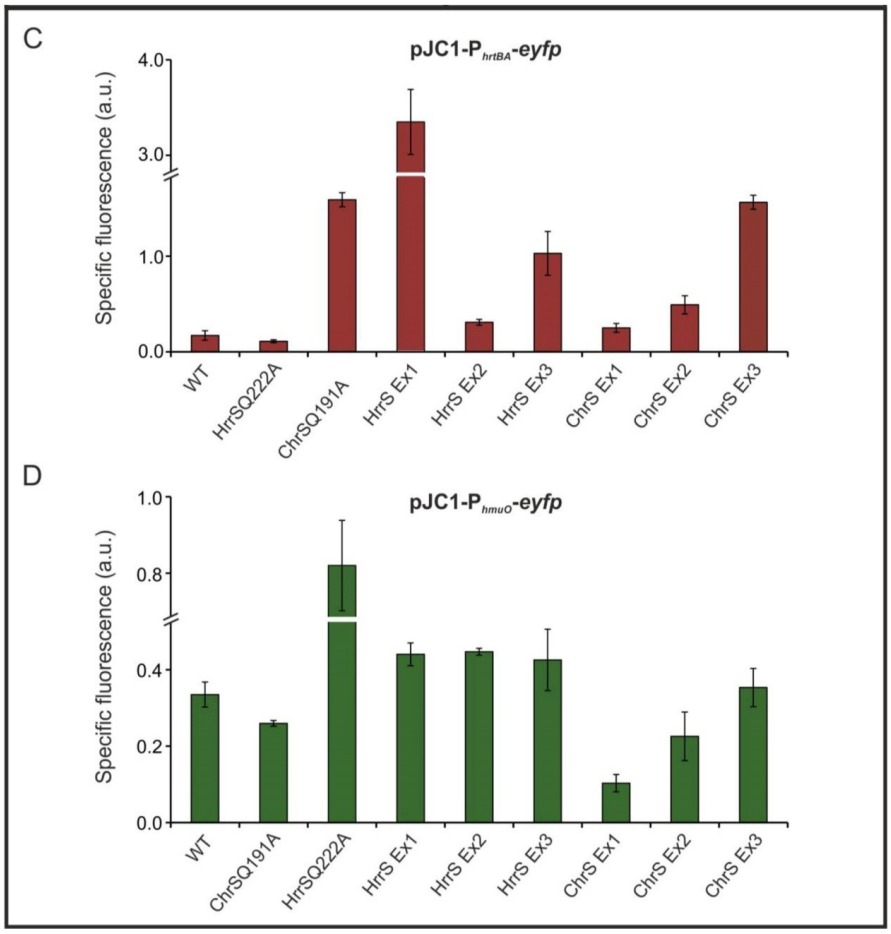

Figure 6: Dissection of the of the phosphatase interface. Chimera strains (see Fig. 5 for details), phosphatase mutant strains (HrrSQ222A and ChrSQ191A) and the *C. glutamicum* ATCC13032 wildtype strain (WT) carrying the reporter plasmids pJC1-P_{hmuO}-*eyfp* and pJC1-P_{hrtBA}-*eyfp* were cultivated in the BioLector system in CGXII minimal medium with 2% (w/v) glucose in microtitre plates in the presence of 2.5 µM haem. The eYFP fluorescence (excitation 510 nm/emission 532 nm) was monitored. The specific fluorescence was calculated as fluorescence signal per backscatter signal (given in arbitrary units, a.u.). A. The P_{hrtBA} activity of the different strains recorded after 2.5 h is shown; the background fluorescence of strains carrying the empty vector control pJC1 was subtracted from all signals. B. The P_{hmuO} activity recorded after 10 h in the indicated strains is presented. The background fluorescence was substracted as described above. Representative experiments of three independent biological replicates are shown.

the phosphatase OFF strains were cultivated in the BioLector in CGXII minimal medium in the presence of 2.5 µM haem. The reporter fluorescence was monitored after 2.5 h (P_{hrtBA}-*eyfp*) and 10 h (P_{hmuO}-*eyfp*), respectively (Fig. 6).

In the following, the results for the chimera strains were the ChrS DHp domain or parts of this domain were exchanged by those of HrrS (HrrSQ222A HrrSEx1-Ex3) to switch specificity from ChrS to HrrA will be described (Fig. 6A and

B). As in the phosphatase OFF strain (10-fold upregulation in contrast to wildtype) these chimera strains displayed an upregulation of the P_{hrtBA}-*eyfp* reporter signal in comparison to the wildtype (Fig. 6A). An exchange of the complete DHp domain of ChrS by those of HrrS even leads to a 20-fold upregulation of the target gene reporter in contrast to the wildtype (2-fold in contrast to HrrSQ222A). This upregulation is due to the complete loss (HrrS Ex1) of the ChrS DHp domain, disrupting the interface crucial for phosphatase activity of ChrS for ChrA. Hyperphosphorylated ChrA then leads to an increased activation of the target gene reporter. But also the exchange of parts of the ChrS DHp domain leads to an upregulation of the target gene reporter P_{hrtBA}-*eyfp*. (HrrS Ex2: 2-fold and HrrS Ex3: 6 fold upregulation). The reason for the higher signal in the chimera HrrS Ex3 in contrast to HrrS Ex2 might have stuctural reasons.

The chimera strains described above were further used for the investigation of the HrrSA target gene reporter P_{hmuO}-*eyfp* to monitor the switch of the interface from ChrS to HrrA (Fig. 6B). In contrast to the wildtype, the phosphatase OFF strain HrrSQ222A showed a 3-fold upregulation of the target gene reporter. An exchange of the complete ChrS DHp domain or parts thereof by those of HrrS (HrrS Ex1 – Ex3), leads to a 2-fold reduction of the signal for all chimera strains in contrast to the phosphatase OFF strain HrrSQ222A (1.3-fold higher in contrast to wildtype). The phosphatase interface could be switched from ChrS to HrrA, but from the different chimeras, we can not conclude the localization of the interface residues, because the effects are identical for all strains (Fig. 6B).

In the following section the chimera strains were the HrrS DHp domain and parts thereof were exchanged by the corresponding areas of HrrS will be described. Investigation of the target gene reporter P_{hrtBA}-*eyfp* in the chimera strain ChrS Ex1 – Ex3 in the strain background ChrSQ191A, revealed a stepwise downregulation of the high signal observed for ChrSQ191A (Fig. 6A). An exchange of the non conserved region from the C-terminus of the first- to the N-terminus of the second α-helix did not result in a lower signal in contrast to ChrSQ191A, meaning, these amino acids are not sufficient to switch the phosphatase interface of HrrS to ChrA (Fig. 6A). In comparison to that, ChrS Ex2 and Ex1 displayed lowered reporter output in contrast to the ChrSQ191A strain (3-fold and 6-fold reduced), which was comparable to the wildtype for ChrS Ex1 (Fig. 6A). This indicates that interface residues, crucial for conferring phosphatase specificity are spread over the complete ChrS DHp domain and not concentrated on the C-terminus of the first and the N-terminus of the second α-helix.

Investigation of this target gene reporter P_{hmuO}-*eyfp* in the chimera strain ChrS Ex1 – Ex3 in the strain background ChrSQ191A, displayed a lower signal for ChrS Ex1 and Ex2 in contrast to the wildtype and ChrS Ex1 showed a signal comparable to the wildtype. As the putative interface of Hrrs was exchanged by the residues of ChrS, we expected an upregulation of the P_{hmuO}-*eyfp* reporter, because of the expected phosphatase interface disruption.

Discussion

The first step of the establishment of new signalling pathways often involves gene duplication events followed by an immediate divergence of the identical TCS. Considering the large number of TCS harboured by some bacterial species and their homology, there is an obvious potential for cross-talk (Capra and Laub, 2012, Yamamoto *et al.*, 2005). But maintaining the correct flux of information in TCS signalling processes is indispensable for bacterial survivial. Several mechanisms have been described to enforce signal transduction specificity in TCS

signalling, among them phosphatase activity and molecular recognition (Podgornaia and Laub, 2013, Salazar and Laub, 2015). Recently it was shown, that the haem-dependent TCS HrrSA and ChrSA from *C. glutamicum* exhibit a high level of cross-talk, which was counteracted by a highly specific phosphatase activity of HrrS and ChrS. However the identified catalytical phosphatase motif turned out to be identical for Hrrs and ChrS (DTVAQ) (Hentschel *et al.*, 2014). Consequently, the question arises if molecular recognition plays a further role in insulation of HrrSA and ChrSA signal-transduction during the phosphatase reaction (Hentschel *et al.*, 2014). It might be feasible that catalytical phosphatase residues are identical and only the interface changed during the course of evolution. Thus, this study aimed at the identification of further catalytical phosphatase residues and to contain the localization of interface residues crucial for the highly specific phosphatase reaction of HrrS and ChrS.

Phylogeny and DHp domain analysis of *C. glutamicum* kinases

In the genome of *C. glutamicum* 13 TCS can be found (Bott and Brocker, 2012). Phylogenetic analysis of the *C. glutamicum* HKs revealed a representation of two subgroups, the HisKA and HisKA_3 type kinases, which were presumably derived from one common anchestor *via* gene duplication events (Fig. 1A). Gene duplication was also described to be driving force for the expansion of the 140 kinases found in *Streptomyces coelicolor*, whereas *E. coli* and *Bacillus subtilis* tend to aquire new TCS by horizontal gene transfer (Alm *et al.*, 2006).

In the present study, a motif search analysis of the DHp domain of *C. glutamicum* kinases was performed. Here, two different conserved phosphatase motifs in close proximity to the conserved histidine residue could be identified for the two subgroups HisKA and HisKA_3 (Fig. 1B). The motif ExxT semms to be highly conserved for HisKA type kinases and a DxxxQ motif (except for CgtS6 - DxxxR) appeared to be highly conserved for HisKA_3 type kinases. This is consistent with prior findings, demonstrating the importance of such a DxxxQ motif for catalytical phosphatase activity of HisKA_3 type kinases (Huynh *et al.*, 2010). Furthermore it was shown that HisKA domain motif E/DxxT/N motif plays a similar role for phosphatase activity of HKs (Huynh and Stewart, 2011, Willett and Kirby, 2012). Recently this was demonstrated for WalK from *Bacillus anthracis* (Dhiman et al., 2014). All of the *C. glutamicum* HKs harbour such a conserved phosphatase motif inside their DHp domains suggesting that all HKS might be bifunctional and the conserved phosphatase motifs as a general mechanism for catalytical phosphatase activity in both HK subfamilies in *C. glutamicum*.

DHp domain architecture of haem-dependent TCS

To gain a first overview of the DHp domain conservation of HrrS and ChrS an alignment of the DHp domain of different *Corynebacterial* species was performed. Here, two hotspots of conserved residues could be identified (Fig. 2A). The first hotspot was located inside the N-terminal region of the first α-helix forming the H-box (ERQRIAHEI**HDTVAQ**GLSSIQMLL). Within this conserved area, the conserved histidine-residue, in close proximity to the phosphatase domain can be found (indicated in bold). The second motif can be found in the N-terminal region of the second α-helix (LARxTAADNL). This second α-helix includes the X-box region with its hydrophobic amino acids, reponsible for the dimerization of the HKs (Grebe and Stock, 1999). A relatively small subset of non-conserved residues in the interface between HisKA type HKs and RRs was shown to dictate specificity upon HK and RR interaction. These residues were shown to be located the DHp domain of

the HisKA type HK and the REC domain of their cognate RR (Skerker *et al.*, 2008, Laub and Goulian, 2007, Capra *et al.*, 2012). An aligment of the HrrS/ChrS DHp and HrrA/ChrA REC domain revealed a high sequence similarity and only few residues in the helices of these domains are not conserved (Fig. 2B). These might be involved in the formation of the interface, playing a critical role in conferring phosphatase specificity. Especially in the first α-helix of the HrrA and ChrA REC domain an accumulation of hydrophobic amino acids can be observed. Prior studies mentioned that these hydrophobic residues might be important for the interface formation *via* Van-der-Waals forces and hydrophobic interactions (Podgornaia and Laub, 2013).

Identification of putative catalytical phosphatase residues

To identify further catalytical phosphatase residues, a HrrS DHp error prone library was constructed and a screening for kinase ON/phosphatase OFF mutants was performed (Fig. 3 and 4). A FACS screening of the library confirmed the glutamine residue (Q222R) inside the DTVAQ phosphatase motif as key residue for the catalytical phosphatase activity of HrrS. Interestingly clones carrying this specific mutation were isolated with the highest frequency. The conserved glutamine residue was described to be crucial for catalytical phosphatse activity of the HisKA_3 subfamily in several studies, for example for phosphatase activity of LiaS from *Bacillus subtilis* or NarX from *E. coli* (Huynh *et al.*, 2010, Schrecke *et al.*, 2013). Furthermore, a threonine residue (T219R), which is also part of the conserved HrrS phosphatase motif, could be identified. Moreover, a cluster of amino-acids (S225, I227, Q228, M229 and H232) which are all located within the H-Box of the HrrS DHp domain turned out to be putative candidates for further catalytical residues, as these are highly conserved residues among haem-dependent TCS (Fig. 2A and 4). For the HisKA subfamily it was shown that only one residue (T/N) within the highly conserved motif H-E/D-x-x-T/N is required exclusively for phosphatase activity in *Myxococcus xanthus* and *Thermotoga maritima* (Huynh *et al.*, 2013). To prove the relevance of the identified residues for catalytical phosphatase activity *in vitro* phosphorylation assays with purified HrrS have to be performed, to demonstrate a diminished phosphatase activity and asses the impact of these mutations on kinase activity. Protein-protein interaction studies will further reveal if these mutations might lead to a loss in affinity, to distinguish between catalytical and interface might lead to a loss in distinguish between catalytical and interface residues.

Partner recognition during phosphatase reaction

To identify interface residues conferring specificity during phosphatase reaction, chimera strains of the HrrS and ChrS DHp domain were constructed (Fig. 5) to redirect the phosphatase specificity for the non- cognate partner in a phosphatase OFF background strain (Fig. 6). Phosphatase specificity could be redirected from HrrS to ChrA by exchanging the complete DHp domain of HrrS by the corresponding region of of ChrS, as the P_{hrtBA}-*eyfp* reporter displayed a wildtype-like signal in the ChrSQ191A ChrSEx1 chimera strain (Fig. 6A). *Vice versa* an exchange of parts or the complete DHp domain of ChrS by the corresponding regions of HrrS led to an increased P_{hrtBA}-*eyfp* reporter fluorescent output, as ChrS is lacking its interface and thus not able to discriminate and dephosphorylate its cognate RR ChrA. This confirms the importance of the DHp domain of ChrS for the formation of the interface during phosphatase reaction. Several studies reported on interface residues conferring specificity in TCS signalling. The crystal structure of the TCS complex HK853-

RR468 from Thermotoga maritima revealed that interface residues conferring specificity are mainly located in the first α-helix of the RR REC domain and form a complex with both α-helices of the HK DHp domain (Podgornaia *et al.*, 2013). Controversially, phosphatase activity could not be completely switched from ChrS to HrrA (HrrS Ex1- Ex3, Fig. 6B) in the present study. Specificity from ChrS to HrrA could probably not be redirected because chimera construction might lead to structural perturbations of ChrS affecting phosphatase activity. Similary the P_{hmuO}-*eyfp* signal in the ChrSEx1-Ex3 chimera strains, where the HrrS DHp domain and parts thereof were exchanged by corresponding regions of ChrS did not ultimately lead to an increase in fluorescence although a disruption of the HrrS phosphatase activity was expected. Hence, it is possible that the domain exchange affected HrrS kinase activity. Furthermore it is possible, that the chimera level is lower than those of the original and cognate partner and that the stoichiometry also has an impact here. As domain exchanges did not always result in the redirection of specificity and caused structural perturbations, mutual information score analysis could be another valuable tool to pinpoint HrrS and ChrS residues forming the interface during phosphatase reaction (Szurmant and Hoch, 2010). Selected HK chimeras will be further analysed regarding their interaction with the cognate and non-cognate RRs. Binding affinities of purified proteins will be determined using protein-protein interaction studies and *in vitro* phosphorylation assays will be performed to further asses the catalytical phosphatase activity.

The homologous TCS HrrSA and ChrSA represent an ideal model to study the role of molecular recognition during phosphatase activity as they show a high level of cross-talk, respond to the same haem stimulus and phosphatase activity is specific only for the cognate RR (Hentschel *et al.*, 2014).

Our study provides first evidence for the localization of interface residues conferring phosphatase specificity of ChrS within the DHp domain. Furthermore putative catalytical phosphatase residues of HrrS could be identified. This lays the groundwork for the dissection of the phosphatase interface of the TCS HrrSA and ChrSA.

Experimental procedures

Bacterial strains and growth conditions

As wildtype strain *Corynebacterium glutamicum* ATCC 13032 (Kalinowski, 2005) and for cloning purposes *E. coli* DH5α was used. *E. coli* was cultivated in Lysogeny Broth (LB) medium at 37°C or on LB agar plates. When necessary, 50 µg ml $^{-1}$ kanamycin was added. For reporter assays and FACS screening experiments, *C. glutamicum* cells were inoculated from a BHI pre-culture (brain heart infusion, DifcoTM BHI, BD, Heidelberg, Germany) and cultivated in CGXII minimal medium (Keilhauer *et al.*, 1993) respectively. The media contained 2% glucose as a carbon and energy source and 2.5 µM (protoporphyrin IX with Fe^{3+}) as an iron source. When necessary, 25 µg ml $^{-1}$ kanamycin was added. The bacterial strains used in this study are listed in Table 1.

Cloning techniques

Cloning was performed according to standard protocols (Sambrook *et al.*, 2001). Chromosomal DNA of *C. glutamicum* ATCC 13032 was prepared (Eikmanns *et al.*, 1994) and utilized as a template for PCR. DNA sequencing and oligonucleotides synthesis were performed by Eurofins MWG Operon (Ebersberg, Germany). Plasmids and oligonucleotides used in this work are listed in Tables S1 and S2 respectively. In-frame deletion mutants of *hrrS*/*chrS* (cg3248, cg2201) and chimeras were constructed *via* the two-step homologous recombination method as described before (Niebisch and Bott, 2001, Hentschel *et al.*, 2014).

Site-directed mutagenesis

For mutagenesis of the conserved DxxxQ phosphatase motif HrrS-Q222A and ChrSQ191A amino acid exchanges were generated *via* sitedirected mutagenesis as described before (Hentschel *et al.*, 2014).

Chimera construction

For construction of chimeras, here exemplified for HrrSEx1 (see also Fig. 3A) 240 bp of the corresponding upstream and in a second step 240 bp of the corresponding downstream region of the *chrS* DHp domain (encoding for ChrS 96-176 and ChrS 243-323) was amplified with primers ChrS-1-fw/ChrS-2-v and ChrS-3-fw/ChrS-4-rv, respectively. In this amplification step 20 bp overlaps to the pK19mobsacB vector (at the 5 prime end of the *chrS* upstream and the 3 prime end of the *chrS* downstream fragment) and to the *hrrS* chimeric domain (at the 3 prime end of the *chrS* upstream and the 5 prime end of the *chrS* downstream fragment) were introduced. In a third step the DHp domain of HrrS encoding for the protein sequence HrrS 208-281 was amplified using the primers HrrS-Ex1-fw and HrrS-Ex1-rv, thereby introducing 20 bp overhangs to the *chrS* fragments described above (oligonucleotides are listed in Table S2). Subsequently, the three fragments of respective genes and the BamHI/EcoRI linearized pK19mobsacB vector were fused *via* the 20 bp overlaps *via* Gibson cloning (Gibson *et al.*, 2009). The pK19mobsacB inserts for other chimeras were created analogously (see Fig. S2).

The resulting plasmids pK19mobsacB-*hrrS*Ex1-3 and pK19mobsacB-*chrS*Ex1-3 (plasmids listed in Table S1) were used for the construction of *C. glutamicum* chimera strains by homologous recombination as described previously (Schäfer *et al.*, 1994). As initial strains the *C. glutamicum* phosphatase OFF strains HrrSQ222A and ChrSQ191A were used. Successful chimera strain construction was verified by colony PCR followed by DNA sequencing.

The *C. glutamicum* wild type, deletion and chimera strains were transformed with the resulting plasmids according to a standard protocol (van der Rest *et al.*, 1999). The construction of promoter fusions of the promoters of *hrtBA* and *hmuO* with *eyfp* was described previously (Heyer *et al.*, 2012).

Error prone library construction

For the identification of further catalytical residues involved in phosphatase activity of HrrS, error prone libraries of the HrrS full lenght DHp domain (HrrS-DHp library) and the left part of the HrrS DHp domain (HrrS-DHp-left library), covering the highly conserved H-Box were constructed. In a first step the *hrrS* DHp domain (encoding for HrrS 208-281) and the left part of the *hrrS* DHp domain, covering the highly conserved H-Box (encoding for HrrS 208-232) were amplified *via* Error-prone PCR using the primers HrrSep-DHp-fw/HrrSep-DHp-rv and the primers HrrSep-DHpleft-fw/HrrSep-DHpleft-rv, respectively. Error-prone PCR was performed using the Diversify kit (Clonetech, Saint-Germain-en-Laye, France). Conditions were chosen to introduce a single amino acid substitution per protein, as given in the supplier's manual. In a second step, the corresponding up- and downstream regions of *hrrS* including the promoter region (200 bp upstream of start ATG) were amplified via PCR using the Primers HrrS-DHp-1-fw/HrrS-DHp-2-rv and HrrS-DHp-3-fw/HrrS-DHp-4-rv (for construction of HrrS-DHp library) and HrrS-DHp-left-1-fw/HrrS-DHp-left-2-rv and HrrS-DHp-left-3-fw/HrrS-DHp-left-4-rv (for construction of HrrS-DHp-left library), thereby introducing 20 bp overhangs to the respective error prone fragments and to the pJC1 vector (oligonucleotides are listed in Table S2). Subsequently, both fragments and the BamHI/SalI linearized pJC1 vector were fused *via* the 20 bp overlaps *via* Gibson cloning (Gibson *et al.*, 2009) resulting in the plasmid libraries pJC1-*hrrS*-DHp and pJC1-*hrrS*-DHp-left.

For library construction, electrocompetent *E. coli* DH5α cells (Invitrogen, Darmstadt) were used. Cells were regenerated in LB medium and after regeneration inoculated in 15 mL of LB medium containing 50 µg mL^{-1} of kanamycin. After overnight cultivation, plasmid libraries were isolated from these cultures. As control, a pJC1-*hrrS* vector, expressing the wild-type HrrS protein was used. Therefor, *hrrS* including the promoter region (200 bp upstream) was amplified via PCR using the primers HrrS-DHp-1-fw and HrrS-DHp-4-rv. Cloning of pJC1-*hrrS* was performed as described above.

To enable screening of the *hrrS* DHp mutant libraries for further catalytical phosphatases residues a genomically integrated P_{hmuO}-*venus* reporter was used. For the promoter fusion of *hmuO* and *venus*, the *hmuO* upstream region was amplified by using the oligonucleotides PhmuO-fw and PhmuO-RBS-rv, while *venus* was amplified with the oligonucleotides Venus-RBS-fw and Venus-rv, thereby introducing 20 bp overhangs between the fragments and to the pK18mobsacB vector (oligonucleotides are listed in Table 3). Subsequently, both fragments and the XhoI/MfeI linearized pK18mobsacB vector were fused via the 20 bp overlaps *via* Gibson cloning (Gibson *et al.*, 2009). The resulting plasmid pk18-P_{hmuO}-*venus* was used for integration of the P_{hmuO}-*venus* reporter into the intergenic region between cg1121/cg1122 in the strain Δ*hrrS*/Δ*chrS*.

FACS and Library Screening.

To enable sorting, *C. glutamicum* Δ*hrrS*/Δ*chrS*-cg1121/cg1122:: P_{hmuO}-*venus* was transformed with the pJC1-*hrrS*-DHp (library size: ~10^5) and pJC1-*hrrS*-DHp-left (library size: ~5 x 10^4) libraries. Cells were regenerated in BHI complex medium (Difco Laboratories Inc., Detroit, MI, USA) for 1 h at 30 °C and plated on BHI agar plates containing 15 µg mL^{-1} of kanamycin. Plates were incubated for 48 h at 30 °C and swept with 2 mL of CGXII without iron/+PCA containing 2 % glucose. The cell suspensions were stored as cryostocks containing 20% glycerol (w/v).

Prior to FACS screening, the CGXII precultures without iron/+PCA containing 2 % glucose and 25 µg mL^{-1} of kanamycin were inoculated from the cryostocks and then grown overday, before being used to inoculate the main culture. The CGXII main cultures, containing 2.5 µM haemin were grown overnight up to OD_{600} of 20. Approximately 1 ×10^5 cells from strains, expressing HrrS-DHp and HrrS-DHp-left libraries were subjected to the FACS analysis. Cells emitting high fluorescence were spotted directly on BHI agar plates containing 25 µg mL^{-1} of kanamycin. Immediately prior to FACS analysis, the cell suspensions were diluted to an optical density below 0.1 and analyzed by a FACS ARIA II high-speed cell sorter (BD Biosciences, Franklin Lakes, NJ, USA) using excitation/emission wavelength of 488/ 530 ± 20 nm and a sample pressure of 70 psi.

Data were analyzed using BD DIVA 6.1.3 and FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR 97520). The electronic signal threshold was defined to exclude nonbacterial particles on the basis of forward versus side scatter areas. Electronic gating in the EYFP channel was set to exclude nonfluorescent cells. Noise level was defined by Δ*hrrS*/Δ*chrS*-cg1121/cg1122:: P_{hmuO}-*venus C. glutamicum* cells, containing a pJC1-*hrrS* vector, expressing the wild-type HrrS protein. Spotted cells were grown for 2 days at 30 °C. Afterwards, colony PCR was performed using the primers HrrS-Seq-fw and HrrS-Seq-rv. For gate P2, 50 clones of each library and for gate P1, 100 clones per library were sequenced (Eurofins MWG Operon Ebersberg, Germany).

Reporter assays in microtitre scale

For reporter assays a 20 ml pre-culture of CGXII minimal medium containing 2% (w/v) glucose was inoculated from a 5 ml BHI culture after washing the cells with 0.9% (w/v) NaCl. To adjust *C. glutamicum* to iron starvation

conditions, no iron source (haem or $FeSO_4$), but PCA was added to the second pre-culture, allowing the uptake of trace amounts of iron present in the growth medium. Cells were incubated overnight at 30°C and 120 r.p.m. in a rotary shaker and grew to an OD_{600} of ~ 20. For all reporter assay experiments, PCA was added to the CGXII minimal medium. Reporter assays in microtitre scale were performed in the BioLector system (m2p-labs GmbH, Aachen, Germany). Therefore, 750 µl CGXII medium containing 2% (w/v) glucose and 2.5 µM haemin were inoculated from the second pre-culture with iron-starved cells (0 µM $FeSO_4$ + PCA) to an OD_{600} of 1 and cultivated in 48-well Flowerplates® (m2p-labs GmbH, Aachen, Germany) at 30°C, 95% humidity, 1200 r.p.m. and a shaking diameter of 3 mm. The haemin solution was added from a sterile filtered stock solution after autoclaving, as indicated. For the haemin stock solution, haemin (Sigma Aldrich, Munich, Germany) was dissolved in 20 mM NaOH to a concentration of 250 µM. When necessary, 25 µg ml^{-1} kanamycin was added. The production of biomass was determined as the backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor of 12); measurements were taken in 10 min intervals. For promoter fusion studies the eYFP chromophore was excited at 510 nm and emission was measured at 532 nm (signal gain factor of 50). The specific fluorescence (arbitrary units, a.u.) was calculated as the ratio of the eYFP fluorescence signal and the backscatter signal (Kensy *et al.*, 2009). To correct for background fluorescence e.g. of the media, fluorescence of respective strains carrying the empty vector as negative control was subtracted from all measurements.

Sequence analysis

Amino acid sequence alignments and phylogenetic analysis were performed using the online tool ClustalW2 (Larkin *et al.*, 2007) and Jalview (Waterhouse *et al.*, 2009). Motif analysis were performed using the MEME Suite tool (Bailey *et al.*, 2009). Prediction of domains was performed with the help of Pfam database (Finn *et al.*, 2014). Secondary structure prediction was implemented with the PSIPRED3 program (Jones, 1999).

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4 Discussion

4.1 Control of haem-homeostasis in *C. glutamicum*

Iron is an essential trace element for almost all bacteria as it serves as cofactor in many proteins such as enzymes involved in the TCA cycle, electron transport or DNA biosynthesis (Skaar, 2010). Besides iron, haem represents an attractive alternative source of iron. Especially pathogens acquire haem and iron from host iron sources including transferrin, lactoferrin, and haemoglobin (Runyen-Janecky, 2013, Nobles and Maresso, 2011, Otto *et al.*, 1992).

Survival in changing environments, like the soil or a human host, critically relies on the ability of a species to sense and adapt to the particular conditions. The most prominent mode of perception and transduction of extra- and intracellular stimuli are two-component systems (TCS) (Stock *et al.*, 2000). After their discovery over 30 years ago great progress in the identification and unravelling of the function of numerous TCS has been made (Ferrari *et al.*, 1985, Nixon *et al.*, 1986). TCS enable the sensing and adaptation of the cellular physiology to various intra and extracellular stimuli, among them stress stimuli, osmolarity changes, antibiotics, the redox state, quorum signals, and nutrients (Calva and Oropeza, 2006, Steele *et al.*, 2012). In the present work we focused on the characterization of the haem-dependent TCS ChrSA from *C. glutamicum* and uncovered a close interplay with the homologous TCS HrrSA. Both TCS HrrSA and ChrSA allow the utilization of haem as an alternative source of iron in the Gram-positive soil bacterium *C. glutamicum*.

C. glutamicum and its close pathogenic relative *C. diphtheriae* both use the ABC-transporter HmuTUV and surface exposed haem-binding proteins (e.g. HtaA) for haem uptake (Frunzke *et al.*, 2011, Allen and Schmitt, 2009, Allen and Schmitt, 2011). After entering the cell, haem is degraded by the haem oxygenase HmuO, resulting in the release of iron. The haem-dependent activation of *hmuO* expression was recently shown to be mediated by the TCS HrrSA in *C. glutamicum* (Frunzke *et al.*, 2011). In the presence of iron, expression of *hrrA* itself, the haem importer *hmuTUV*, and the haem oxygenase *hmuO* is repressed by the global iron regulator DtxR (Frunzke and Bott, 2008, Wennerhold and Bott, 2006, Wennerhold *et al.*, 2005). However the utility of sufficient haem supply is inseparable from its toxicity. One possible scenario is that

iron, released during the degradation of haem can be involved in the formation of reactive oxygen species *via* Fenton reaction leading to DNA damage (Everse and Hsia, 1997). Based on the fact that bacteria have evolved sophisticated strategies to avoid haem toxicity e.g. the export, another explanation is, that haem itself can be toxic by an unknown mechanism (Stojiljkovic *et al.*, 1999). Thus, utilization and detoxification of haem have to be tightly regulated by bacteria.

In this previous work a second TCS termed ChrSA appeared to be a further target of HrrSA (Frunzke *et al.*, 2011). Thus, the role of ChrSA as a second key player in the control of haem homeostasis was characterized during this study. The TCS mediated control of haem homeostasis seems to be a general mechanism for bacteria, but whereas some bacteria expend only one TCS as *C. urealyticum* or *C. ulcerans*, others hold even two TCS as regulators for the haem-homeostasis, as it is the case for *C. glutamicum* and *C. diphtheriae* (Bibb and Schmitt, 2010, Bott and Brocker, 2012, Tauch *et al.*, 2008, Trost *et al.*, 2011). In *C. glutamicum* HrrSA and ChrSA share a high sequence similarity with each other and respond to the same stimulus (haem), consequently the question appeared if both TCS can interact with each other. For this reason the major focus of this work was to uncover the level of interaction between the TCS HrrSA and ChrSA and to disclose mechanisms ensuring pathway specificity in these highly intertwined TCS.

4.1.1 The two-component system ChrSA is crucial for haem-detoxification

The TCS ChrSA was described to be crucial for conferring resistance against high levels of haem in many Gram-positive species (Joubert *et al.*, 2014, Bibb and Schmitt, 2010). In the present study the TCS ChrSA was shown to activate the expression of the divergently located operon *hrtBA*, encoding the ABC-transporter HrtBA, which is crucial for the export of haem. Deletion mutants of *hrtBA* and *chrSA* displayed a high sensitivity towards haem underlining the importance of the TCS ChrSA for the detoxification of haem (Heyer *et al.*, 2012). Besides that, reporter assays pointed towards a contribution of ChrSA in the activation of the expression of *hmuO* which is mainly activated by the TCS HrrSA (Frunzke *et al.*, 2011, Heyer *et al.*, 2012).

The orthologous TCS ChrSA from *C. diphtheriae* shares a high sequence similarity with ChrSA from *C. glutamicum* (ChrS: 29%, ChrA: 44%) and also activates the expression of *hrtBA*. However, in contrast to *C. glutamicum*, in *C. diphtheriae* the TCS

ChrSA is also the main activator of *hmuO* expression as it contributes to 80 % of the activation (Bibb *et al.*, 2005, Bibb *et al.*, 2007). Furthermore, in *C. diphtheriae* ChrSA represses together with HrrSA the expression of *hemA*, which is involved in haem-biosynthesis, which is also the case for *C. glutamicum* (Bibb *et al.*, 2007, Frunzke *et al.*, 2011). The present study shows that in *C. glutamicum* HrrSA and ChrSA inherit distinct roles in the control of haem utilization and detoxification on the other hand, whereas in *C. diphtheriae* this role is mainly taken over by ChrSA (Heyer *et al.*, 2012, Bibb *et al.*, 2007). However, the regulation of target genes by ChrSA and HrrSA in *C. diphtheriae* was not investigated under different conditions which could also have an influence on the contribution of the systems regarding their target gene activation.

Such haem detoxification systems are widespread among Gram-positive bacteria, especially in pathogens and saprotrophs a high conservation can be observed (Stauff and Skaar, 2009b). Orthologous systems comprised by the haem exporter HrtBA regulated by a TCS (HssRS) could be identified in *Staphylococcus aureus*, *Stapylococcus epidermis*, *Bacillus cereus*, *Bacillus subtilis*, *Bacillus anthractis*, *Listeriae monocytogenes* and many other (Stauff and Skaar, 2009b, Torres *et al.*, 2007, Juarez-Verdayes *et al.*, 2012).

Further strategies to overcome the toxicity of haem besides simply exporting it are the sequestration and degradation of haem. *Plasmodium ssp.* the causative agent of malaria, seuquesters haem in a nontoxic insoluble substance termed hemozoin. This reaction is catalyzed by the haem detoxification protein (HDP) (Fitch, 1998, Jani *et al.*, 2008). Another example for haem sequestration is the haem binding protein family HemS. In *Yersinia enterocolitica* deletion of *hemS* resulted in an increased sensitivity towards increased haem levels (Stojiljkovic and Hantke, 1994). Prevention of haem toxicity was also considered to be mediated by degradation. In mammals, a haem oxygenase family of monooxygenases functions was first identified to protect cells from the deleterious effects of haem (Poss and Tonegawa, 1997). The wide distribution of such haem detoxification mechanisms among diverse species highlights their importance and their need for a tight control.

The existence of a TCS in combination with an exporter to prevent toxicity of various substances is a common phenomenon. For *Bacillus subtilis* it was recently described that the TCS BceRS confers resistance towards bacitracin as it activates the

expression of a haem exporter *bceAB*. Interestingly, in this case, the transporter constitutes the bacitracin sensor and communicates this to the HK (Dintner *et al.*, 2014). Whether the haem-exporter HrtBA is involved in haem sensing and interacts with ChrSA in *C. glutamicum* could be elucidated in reporter assays with *hrtBA* deletion mutants and protein-protein interaction studies in future experiments. Besides that, until now it is not clear, how haem is detected by HrrS and ChrS in *C. glutamicum*.

For ChrS from *C. diphtheriae* it was suggested that haem sensing occurs in the intracellular space. It was shown that putative residues involved in haem binding are located in the second and fourth transmembrane domain. However, the identified residues were located intra- and extracellular and even in the transmembrane region, which requires additional studies to identify the location of haem detection. Only one mutation of a highly conserved tyrosine residue (Y61) located on the intracellular site of the transmembrane domain, completely abolished ChrS activity in the presence of haem (Bibb and Schmitt, 2010). In contrast to that for *B. anthracis* it was shown during mutation studies of HssS that haem detection occurs either between the membranes and the cell wall or in the membrane itself (Stauff and Skaar, 2009a).

In *C. glutamicum*, reporter studies with a deletion mutant of the haem-importer Δ*hmuTUV* displayed no alteration in signal output (see appendix Fig. S1). This might be due to the hydrophilic nature of haem, entering the cell or the stimulus is detected extracellular. This could be elucidated by mutation of conserved TMD residues and membrane topology analysis in the future.

4.2 Multi-level interaction of the TCS ChrSA and HrrSA

4.2.1 The HrrSA and ChrSA regulon overlap

In our previous work, it was shown that the TCS HrrSA represses the expression of *chrSA*. Furthermore, it was shown, that HrrA is capable of binding to the promoter region of *chrSA* delivering first evidence for an interaction of the TCS HrrSA and ChrSA in *C. glutamicum* on the transcriptional level (Frunzke *et al.*, 2011). *Vice versa* during transcriptome analysis and reporter studies of a *chrSA* deletion mutant and DNA-protein interaction studies, expression of the homologous TCS *hrrSA* appeared to be negatively regulated by ChrSA in the present study (Heyer *et al.*, 2012, Hentschel *et al.*,

2014). Additionally, these studies also demonstrated, that HrrSA and ChrSA control overlapping target genes (Heyer *et al.*, 2012). This is also the case for the dual control of *hemA* and *hmuO* expression by the two orthologous TCS from *C. diphtheriae* as mentioned above (Bibb *et al.*, 2007). In many cases TCS target genes overlap. This was shown for the control of the expression of two porins encoded by *ompF* and *csgD,* which are both controlled by RRs of the TCS EnvZ-OmpR and CpxA-CpxR from *E.coli*, but the regulatory logic behind this is still unclear (Batchelor *et al.*, 2005).

From this study and from prior results it was also shown that even the target gene recognition sites of HrrA and ChrA overlap in *C. glutamicum* (Wolf, 2013, Heyer *et al.*, 2012). Thus it is likely, that both RR might compete for the binding to the target genes and that under a particular condition one RR outcompetes the other for binding. A recent work could demonstrate that this is the case for the orphan RR GlnR and the TCS RR PhoP in *Streptomyces coelicolor*. PhoP and GlnR compete for the binding to the promoter of the target gene *glnA*, but GlnR has a higher affinity to the target sequence. In this case, this allows a modulation of gene expression in response to different stimuli (Sola-Landa *et al.*, 2013). In the case of HrrSA and ChrSA binding affinities to their target genes could be determined using surface plasmon resonance transfer analysis. But as both systems respond to the same stimulus (haem), the physiological role behind this is unclear.

A further potential level of interaction is the heterodimerization of RRs, which was recently shown for two orphan atypical response regulators BldM and WhiI from *Streptomyces venezuelae* controlling morphological differentiation. As homodimer BldM activates several genes involved in the differentiation of aerial hyphae into spores, but as a BldM-WhiI heterodimer, BldM binding specificity to further target genes involved in sporulation is modulated. This allows an integration of different signals and might function as a timing device of gene expression (Al-Bassam *et al.*, 2014). However, as we could not observe an interaction between HrrA and ChrA in bacterial two-hybrid assays (see appendix Fig. S2) a heterodimerization is rather unlikely.

Another possible scenario could be that HrrA and ChrA compete for their binding to target promoters. This could be tested with a plasmid clustering assay developed by Batchelor and Goulian, which enables the visualization of DNA binding of

a RR fused to YFP. Plasmids containing multiple copies of the respective target promoter binding site were used. If RR binding occurs, this leads to a cluster formation, which can be detected *via* fluorescence microscopy (Batchelor and Goulian, 2006). This could be an approach to test a competition between HrrA and ChrA for binding to their target gene promoters under varying RR concentrations. The current model of the regulation of haem homeostasis by HrrSA and ChrSA is summarized in figure 4.2.1.

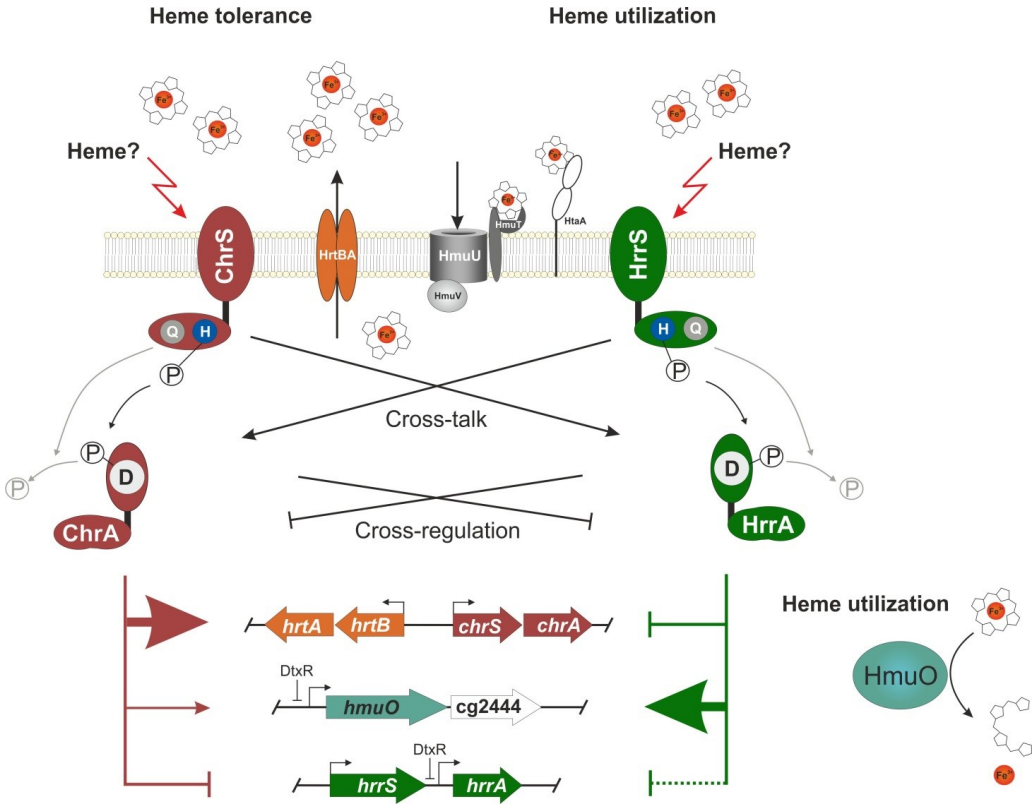

Fig. 4.2.1 Current model of the interaction of the haem-dependent TCS HrrSA and ChrSA in *C. glutamicum*. The TCS ChrSA predominantly mediates detoxification of haem by activation of *hrtBA* upon high haem levels. The HrrSA TCS is required for utilization of haem as an alternative source of iron by activating *hmuO*. Uptake of haem is mediated by HmuTUV/HtaA. Activation of the response regulators ChrA and HrrA is mediated by their cognate kinases ChrS and HrrS in the presence of a stimulus. An interaction of both TCS was observed on the level of cross-talk and cross-regulation. The kinases HrrS and ChrS have a dual function both as kinase and as phosphatase. Phosphatase activity of each kinase was shown to be specific only for the cognate response regulator, thereby ensuring pathway specificity of these closely related systems. When sufficient iron is available, the genes *hrrA* and *hmuO* are both repressed by the regulator of iron homeostasis DtxR. Adapted from (Hentschel *et al.*, 2014, Bott and Brocker, 2012).

4.2.2 HrrSA and ChrSA interact at the level of phosphotransfer

HKs and RRs each comprise paralogous gene families. Some species encode dozens or up to hundreds of these signaling systems (Galperin, 2005, Alm *et al.*, 2006). Thus, these families often share a high similarity regarding their structure and sequence, which provides considerable potential for cross-talk on the phosphorylation level (Fig. 4.2.1). The fact that the TCS HrrSA and ChrSA share a high sequence similarity with each other (HrrS and ChrS: 39%; HrrA and ChrA: 57% identity) and respond to the identical stimulus, suggests that they might also interact at the level of phosphotransfer (Hentschel *et al.*, 2014).

An interaction at the level of phosphotransfer could in fact be observed in *in vivo* and *in vitro* experiments during this study. Mutants lacking the kinase HrrS or ChrS respectively were capable of phosphorylating non cognate RRs. Additionally, this was also demonstrated during *in vitro* phosphorylation assays and in bacterial two-hybrid assays (see appendix Fig. S2) (Hentschel *et al.*, 2014). In *C. diphtheriae* a cross-talk between HrrSA and ChrSA was also suggested, but not tested *in vitro* in prior studies (Bibb *et al.*, 2005, Bibb *et al.*, 2007). In general cross-talk in bacteria can be deleterious and must be avoided to maintain the correct flux of information (Laub and Goulian, 2007). In contrast to that, signal transduction systems in eukaryotes display extensive cross-talk. The kinase Cdk1 for example phosphorylates hundreds of targets in yeast. (Hill, 1998, Ptacek *et al.*, 2005). However, there are a few examples of cross-talk playing a physiological role in bacteria *in vivo*.

One example is the cross-talk between PhoR/PhoP and WalR/WalK (prior named YycG/YycF) from *B. subtilis*, which is due to the physiological relevance termed cross-regulation. Expression of the gene *yocH* is activated by the RR WalK which is phosphorylated by the non-cognate HK PhoR under phosphate limitation (Howell *et al.*, 2003, Howell *et al.*, 2006). Another interesting example of cross-regulation that connects the haem response to cell envelope stress was recently published for *B. anthracis*. Here, the haem responsive ChrSA ortholog HssRS mediates the detoxification of haem as described above (Stauff and Skaar, 2009a). A further TCS HitRS is supposed to be involved in sensing of compounds that alter the cell wall integrity and activates the ABC transporter *hitPQ*. Interestingly, HitRS failed to sense haem. It was shown that HssS phosphorylates HitR *in vivo* and *in vitro*, resulting in an

upregulation of *hitRS* by HssRS in the presence of haem, which allows an integration of signals from different pathways. It was speculated that the connection of sensing haem and detecting perturbations of the cell envelope has a physiological relevance as the hydrophobic haem can accumulate in the membrane at high levels (Mike *et al.*, 2014, Anzaldi and Skaar, 2010).

Physiological relevance of cross-talk between HrrSA and ChrSA in *C. glutamicum* based on the integration of different signals is unlikely, as both TCS respond to the same stimulus (haem). However, spatiotemporal super resolution microscopy analysis of the histidine kinases HrrS and ChrS fused to eYFP revealed that *hrrS* is expressed on a basal level under both iron and haem conditions, whereas *chrS* expression is shut down in the absence of haem and strongly upregulated upon haem supply (unpublished data). These data indicate that cross-talk from HrrS to ChrA, which underlies strong autoactivation, might act as a trigger for *chrSA* expression when the conditions shift towards high haem levels. The physiological relevance of cross-talk could be further investigated in Förster Resonance Energy Transfer (FRET) assays allowing the monitoring of an interaction between HrrSA and ChrSA *in vivo*. Another approach to test cross-talk *in vivo* was developed by Siryaporn and co-workers. Here a fluorescence localization assay where the RRs were fused to YFP was used to monitor HK-RR interaction. Upon HK-RR interaction, RR-YFP comes in close proximity to the HK and is localized to the cell periphery, which can be monitored by fluorescence microscopy (Siryaporn *et al.*, 2010).

4.3 Pathway specificity in HrrSA and ChrSA signal transduction

A relatively new field in TCS research attends to uncover the mechanisms dictating specificity in the concert of bacterial signal transduction (Podgornaia and Laub, 2013, Laub and Goulian, 2007). In eukaryotes cross-talk is often limited by a spatial localization of regulatory proteins, which is mediated by adaptor or scaffold proteins, separating the components from each other (Bhattacharyya *et al.*, 2006). In bacteria several mechanism were described ensuring pathway specificity and avoiding detrimental cross-talk in TCS signal transduction. These mechanisms comprise (i) phosphatase activity of the HKs, (ii) substrate competition, and (iii) molecular recognition *via* specificity residues forming the interface between HK and RR (Laub and

Goulian, 2007). The role of phosphatase activity and molecular recognition for pathway specificity will be discussed in the next sections.

4.3.1 Phosphatase activity ensures pathway specificity of HrrSA and ChrSA

This work delivered striking evidence that phosphatase activity of the histidine kinases HrrS and ChrS is important for conferring specificity in the cross-talking TCS HrrSA and ChrSA in *C. glutamicum*. Mutation of a conserved glutamine residue within a conserved phosphatase motif (DxxxQ) led to an increased signal of target gene reporters, due to a lacking dephosphorylation of RRs. Furthermore, in *in vitro* assays, dephosphorylation was solely observed for the cognate RRs of HrrS and ChrS, respectively (Hentschel *et al.*, 2014).

Phosphatase motifs first of all were identified for auxiliary phosphatases as CheZ and CheX which harbor the phosphatase motifs DxxxQ and ExxN that are crucial for the dephosphorylation of CheY. The conserved residues Gln and Asn both provide an amide group for the orientation of the nucleophilic water and the Asp and Glu residues are capable of forming a salt bridge with a Lys residue of CheY (Pazy *et al.*, 2010, Zhao *et al.*, 2002, Silversmith, 2010).

Phosphatase activity as a determinant of specificity has been described for a number of HKs. Usually the unphosphorylated HK exhibits phosphatase activity in the absence of a stimulus (Stock *et al.*, 2000, Huynh and Stewart, 2011). The theory that the phosphate residue is transferred backwards to the HK was refuted in the past (Huynh and Stewart, 2011). Instead of this, another hypothesis came into focus of researchers. This hypothesis implies that dephosphorylation is catalyzed by the amide or hydroxyl group of a conserved Gln, Asn or Thr residue located within a conserved phosphatase motif in the α1-helix of the DHp domain of HKs (HisKA subfamily: E/DxxN/T, HisKA_3 subfamily: DxxxQ). These residues form a hydrogen bond with a nucleophilic water and orient it for an in-line hydrolysis of the phosphoryl group (Huynh and Stewart, 2011, Huynh *et al.*, 2013). In the present study such phosphatase motifs could also be identified for all of the 13 *C. glutamicum* HKs, which represent the two subgroups HisKA and HisKA_3 underlining the importance of such a dual control.

Phosphatase activity in bacterial TCS signal transduction does not only prevent cross-talk from non-cognate kinases or small phosphate donors like acetyl phosphate,

it also resets the system upon changed environmental conditions (Podgornaia and Laub, 2013). Cross-talk from one of the other 11 TCS from *C. glutamicum* as well as the involvement of small phosphate donors can be excluded for HrrSA and ChrSA, as a deletion mutant lacking both kinases showed an impaired growth and nearly no target gene reporter signal could be observed during the present study (Hentschel *et al.*, 2014).

Prominent examples for the bifunctional nature of HKs of HisKA_3 subfamily include NarX controlling nitrate response from *E. coli*, the LiaFRSR TCS mediating the response towards cell-envelope stress in *B. subtilis* and the membrane fluidity sensor DesK from *B. subtilis*. Consistent with our data, the glutamine residue within the conserved phosphatase motif DxxxQ was also demonstrated to be crucial for conferring catalytical phosphatase activity (Schrecke *et al.*, 2013, Huynh *et al.*, 2010, Albanesi *et al.*, 2004). Investigation of NarX from *E. coli* also suggested an ancillary involvement of the Asp residue for phosphatase activity, here it was proposed that this residue forms a hydrogen bond with a Lys residue of the RR (Huynh *et al.*, 2010). A further study also demonstrated that the CA domain of the HisKA subfamiliy has an influence on phosphatase activity, whereas it has no influence on the phosphatase activity of the HisKA_3 subfamily (Huynh *et al.*, 2013). For several HisKA subfamily members (EnvZ, PleC, CpxA, PhoR, WalK and HK853) it was shown that only one residue (T/N) within the highly conserved motif E/D-x-x-T/N is required exclusively for phosphatase activity, whereas the Glu and Asp residues in close proximity to the histidine are important for kinase activity (Willett and Kirby, 2012, Dutta *et al.*, 2000, Gutu *et al.*, 2010, Hsing *et al.*, 1998, Yamada *et al.*, 1989, Nakano and Zhu, 2001, Raivio and Silhavy, 1997).

Up to this date, the exact mechanism of the differentially regulated phosphatase and kinase activity is not fully understood. With the help of HKs crystal structures some studies suggest that the two modes represent two different conformations and that the rotational movement within the DHp domain helical bundle has an impact on the accessibility of the histidine residues crucial for autophosphorylation and the phosphatase motif (Stewart, 2010, Russo and Silhavy, 1991, Casino *et al.*, 2009, Albanesi *et al.*, 2009).

During our study we started to identify further residues involved in phosphatase activity of HrrS by the establishment of an error prone library of the HrrS DHp domain. During the screening for kinase ON/ phosphatase OFF phenotypes, we were able to isolate further putative phosphatase residues. Interestingly, a highly conserved threonine residue (T219R), which is also part of the conserved HrrS phosphatase motif (DTVAQ) as well as a cluster of amino-acids which are all located within the H-Box of the HrrS DHp domain turned out to be putative candidates for further catalytical residues. The role of these putative phosphatase residues and the impact on kinase activity will be elucidated using *in vitro* phosphorylation assays with purified HrrS in the future. Protein-protein interaction studies will further reveal if these mutations might lead to a loss in affinity, to distinguish between catalytical residues and those forming the interface. Most of the current knowledge about phosphatase activity derives from studies of HisKA family members Investigation of phosphatase activity of HrrS and ChrS would make an important contribution to expand the knowledge of HisKA_3 subgroup phosphatase activity.

4.3.2 Molecular recognition during phosphatase activity of HrrS and ChrS

In this study it was shown that phosphatase activity of HrrS and ChrS is highly specific only for the cognate RR in *C. glutamicum*. However, the catalytical phosphatase motif of HrrS and ChrS turned out to be identical for both (DTVAQ) (Hentschel *et al.*, 2014). Consequently, the question arises if molecular recognition plays a further role in insulation of HrrSA and ChrSA signal-transduction during the phosphatase reaction. It is feasible that catalytical phosphatase residues are identical and only the interface changed during the course of HrrS and ChrS evolution.

The principle of molecular recognition is based on the assumption that a small number of residues enables a HK to discriminate its cognate RR. New TCS pathways typically arise *via* gene duplication events or lateral gene transfer, leading to two identical pathways. For this account, the occurrence of cross-talk is obvious (Alm *et al.*, 2006, Capra and Laub, 2012). In *C. glutamicum* 13 TCS can be found which represent two subgroups HisKA and HisKA_3 (Bott and Brocker, 2012). Phylogenetic analysis of these TCS showed that they probably arised *via* gene-duplication events and share a

common ancestor. Many homologous pairs can be found, which share a high sequence similarity with each other.

To become a new pathway and to avoid the deleterious effects of cross-talk a subsequent divergence after gene duplication is necessary (Capra *et al.*, 2012). Both HK and RR coevolve to maintain their interaction and to become insulated from the ancestral TCS. After specificity is established these residues forming the interface become more or less static (Salazar and Laub, 2015). However, the superordinate principle behind the emergence of new pathways *via* single mutations contributing to specificity is not fully understood. Several studies focused on the identification of interface residues conferring specificity in TCS signalling (Skerker *et al.*, 2008, Laub and Goulian, 2007, Capra and Laub, 2012). These interface residues, being important for the molecular recognition between a HK and its cognate RR are supposed to be located in the first α-helix of the RR REC domain and in both helices of the DHp domain of the HK (Podgornaia and Laub, 2013).

This was also demonstrated for EnvZ from *E. coli*, where residues conferring specificity could be identified. These are mostly located at the C-terminal end of α1-helix and in the the N-terminal part of the α2-helix. Furthermore, they were able to switch specificity from EnvZ to a non-cognate RR RstB by mutating only three residues within the DHp domain and thereby rationally rewired the interface (Skerker *et al.*, 2008). In another important study the co-crystal structure of the TCS HK853-RR468 from *T. maritima* was solved which shed light onto the sequence and structure of the interface conferring specificity (Casino *et al.*, 2009). In a later study the approach of rationally rewiring specificity was used to switch the interaction of HK853-RR468 from *T. maritima* to PhoR-PhoB from *E.coli* (Podgornaia *et al.*, 2013). However, an important contribution to the understanding of the evolution of specificity residues requires the identification of mutational intermediates. For the establishment of a new pathway the intermediates between initial and the final insulated state have to be nearly neutral (Capra and Laub, 2012).

In a recent study, Podgornaia and Laub followed mutational trajectories of the interface of the TCS PhoPQ from *E. coli*. In this work they were able to map the protein interface crucial for molecular recognition and mutated the four key interfacial residues in all possible combinations to screen for functional intermediates. With this

approach they followed mutational trajectories and showed a degeneracy in the interface of PhoPQ and an epistasis which constrains PhoQ evolution (Podgornaia and Laub, 2015).

In the present study, we started with localization studies of residues conferring specificity during phosphatase reaction of HrrS and ChrS in *C. glutamicum*. For these TCS the mechanism of molecular recognition seems to play a greater role during phosphatase activity in contrast to kinase activity. Using chimeric proteins of HrrS and ChrS DHp domains, we obtained first hints that residues conferring specificity are located inside the DHp domains of HrrS and ChrS (for current model see Fig. 4.3.2). However, redirecting phosphatase activity for the non-cognate RR was not possible for all chimeras, as this presumably causes structural perturbations or the stoichiometry might also have an effect here.

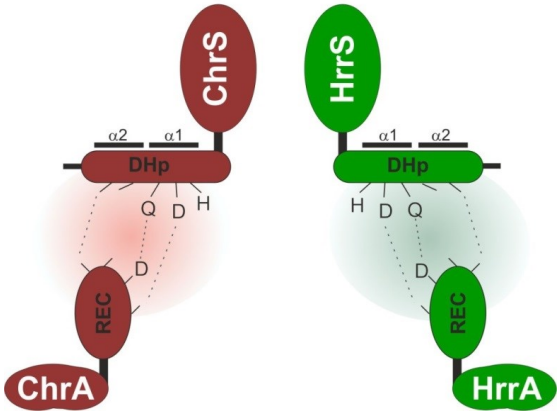

Fig. 4.3.2: Model of the interface of the haem-dependent TCS HrrSA and ChrSA. Schematic representation of the protein-protein interface of HrrSA and ChrSA. Molecular recognition is supposed to be mediated by unknown residues localized in both α-helices of the DHp domain of the HK and in the REC domain of the RR. The conserved histidine residue of HKs HrrS and ChrS is crucial for kinase activity, whereas the conserved glutamine residue was demonstrated to be involved in catalytical phosphatase activity.

Thus, a covariance based computational method termed mutual information score analysis (MIS) could be used to predict residues conferring specificity. This is based on the assumption that every mutation occurring post-duplication in a HK has to be reverted by a further mutation in the cognate RR and *vice versa*. MIS compares the frequency of individual aminoacids at two residue positions in a multiple sequence

alignment to the frequency of the co-occurrence of these aminoacids at the particular residue position (Szurmant and Hoch, 2010). Another approach to identify residues conferring specificity during phosphatase reaction will be a co-crystallization of HrrSA and ChrSA and especially of both TCS with their non-cognate partners.

Thus far, the role of phosphatase activity is controversially discussed. In the study of Siryaporn *et al.* the authors hypothesized that phosphatase activity is strictly the result of an increased HK/RR affinity (Siryaporn *et al.*, 2010). Another recent work focused on the identification of further residues involved in phosphatase activity of NarX, DesK (HisKA_3) and HK853 (HisKA) besides the above described catalytical Gln, Asn or Thr residues. Missense substitutions were created to isolate kinase ON/Phosphatase OFF mutants. Interestingly, they could identify two further types of residues affecting phosphatase activity. These are (i) residues important for conformation and (ii) residues which are crucial for HK and RR specificity that are involved in the formation of the interface. Mutation of these residues resulted in kinase ON/ phosphatase OFF phenotype and in a reduced HK RR affinity (Huynh *et al.*, 2013). This also suggests that phosphatase activity requires a higher affinity than kinase activity.

This is in contrast with the finding that some residues are exclusively required for kinase or phosphatase activity of HisKA subfamily members. In this study a complete dissection of the HisKA domain of the CdrS homologs HK1190 and HK4262 from *M. xanthus* and of HK853 from *T. maritima* was performed. However, mutation of these residues did not result in a lowered partner affinity (Willett and Kirby, 2012). Thus, up to now the role of molecular recognition during phosphatase activity is not clear.

The homolgous TCS HrrSA and ChrSA from *C. glutamicum* represent an ideal model to study the role of molecular recognition during phosphatase activity as they show a high level of cross-talk, respond to the same haem stimulus and phosphatase activity was demonstrated to be highly specific only for the cognate RR in the present study.

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6 Appendix

6.1 Supplementary material – The two-component system ChrSA

Cloning techniques

In-frame deletion mutants of the operons *hrtBA, chrSA,* and *hrrSA* were constructed via the two-step homologous recombination method as described before (Niebisch & Bott, 2001). Therefore, the corresponding upstream region covering the first 30 bp of the gene *hrtB* was amplified by PCR using the oligonucleotides DhrtBA-1and DhrtBA-2. Amplification of the downstream region with the last 30 bp of *hrtA* was performed with the oligonucleotides DhrtBA-3 and DhrtBA-4. Subsequently, the up- and downstream flanking region of *hrtBA* and *chrSA* were fused via an overlap of 21 bp by overlap extension PCR with the oligonucleotides DhrtBA-1/DhrtBA-4 and DchrSA-1/DchrSA-4, respectively. PCR products were ligated into pK19*mobsacB* at the *Bam*HI and *Sal*I restriction sites. The pK19mobsacB inserts for the deletion of chrSA and hrrSA were created analogously using the oligonucleotides DchrSA-1/DchrSA-2 plus DchrSA-3/DchrSA-4 and DhrrSA-1/DhrrSA-2 plus DhrrSA-3/DhrrSA-4, respectively.

The resulting plasmids pK19*mobsacB*-Δ*hrtBA,* pK19*mobsacB*-Δ*chrSA*, and pK19*mobsacB*-Δ*hrrSA* were used for the deletion of the corresponding genes in *C. glutamicum* by homologous recombination as described (Schäfer *et al.*, 1994). Successful deletion was verified by colony PCR and DNA sequencing (oligonucleotides: DhrtBA-fw/DhrtBA-rv, DchrSA-fw/DchrSA-rv, or DhrrSA-fw/DhrrSA-rv respectively).

For complementation of the phenotype of Δ*hrtBA* and Δ*chrSA,* DNA fragments covering the respective operon were amplified with the oligonucleotides hrtBA-RBS-fw/hrtBA-rv and chrSA-fw/chrSA-rv, respectively. The DNA fragment of *chrSA* and its native promoter was cloned after an *Nhe*I digestion, into the low-copy vector pJC1 (Cremer *et al.*, 1990), while the *hrtBA* DNA fragment without the native promoter but with an additional ribosome-binding site was ligated into the *Pst*I restriction site of the pEKEx2 vector under the control of the IPTG-inducible promoter P_{tac}. The *C. glutamicum* wild-type and the deletion strains Δ*hrtBA* and Δ*chrSA* were transformed with the resulting plasmids according to a standard protocol (van der Rest *et al.*, 1999).

For overproduction of ChrA with an N-terminal hexa-histidine tag (addition of 20 amino acids [MGSSHHHHHHSSGLVPRGSH] at the N terminus of the protein ChrA), the coding region *chrA* (cg2200) was amplified by PCR with the oligonucleotides chrA-*Nde*I-fw and chrA-*Hin*dIII-rv thereby inserting *Nde*I and *Hin*dIII restriction sites. The purified PCR product was cloned into pET28b (Novagen) resulting in the vector pET28b-*chrA*.

For the construction of promoter fusions of the promoter of *chrSA* and *hrtBA* with *eyfp,* encoding the yellow fluorescent protein eYFP, 235 bp of the intergenic region of *chrSA* and *hrtBA* were amplified with the oligonucleotides PchrS-*Bam*HI-fw/PchrS-8C-RBS-*Nde*I-rv and PhrtB-*Bam*HI-fw/PhrtB-8C-*Nde*I-rv, respectively, thereby covering the promoter region and the first 24 bp of the respective gene. After 8 codons, a stop codon, a ribosome-binding site and *Bam*HI and *Nde*I restriction sites were inserted by the designed oligonucleotides. As N-terminal peptide-tags may affect protein stability, we used this strategy to translate

A. Heyer, C. Gätgens, E. Hentschel, J. Kalinowski, M. Bott & J. Frunzke. (2012). The two-component system ChrSA is crucial for haem tolerance and interferes with HrrSA in haem-dependent gene regulation in *Corynebacterium glutamicum*. *Microbiology* **158**, 3020–3031.

unaltered eYFP, but to include the first 24 bp of the leaderless transcripts of *chrS* and *hrtB*. The resulting fragments were ligated in front of *eyfp* by exchanging the *brnF* promoter cassette within the vector pJC1-*lrp-brnF'-eyfp* (Mustafi *et al.*, 2012), resulting in the plasmids pJC1-P_{chrSA}-*eyfp* and pJC1-P_{hrtBA}-*eyfp*. For the promoter fusion of *hmuO and eyfp,* the hmuO upstream region was amplified by using the oligonucleotides PhmuO-*Nhe*I-fw and PhmuO-RBS-rv, while *eyfp* was amplified with the oligonucleotides eyfp-RBS-fw and eyfp-*Nhe*I-rv. To combine both fragments a cross-over PCR was performed with PhmuO-*Nhe*I-fw and eyfp-*Nhe*I-frv. The final construct consists of 494 bp of the upstream region, the first 41 codons of the *hmuO* gene followed by a stop codon, a ribosome-binding site and a *Nhe*I restriction site. The cross-over PCR was ligated in the *Nhe*I restriction site of pJC1, resulting in the plasmid pJC1-P_{hmuO}-*eyfp*.

Table S1. Bacterial strains and plasmids used in this study

Strains	Characteristics	Reference
E. coli		
E. coli DH5α	*supE44 ΔlacU169* (ϕ80*lacZ*DM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Invitrogen
E. coli BL21(DE3)	F^- *ompT* $hsdS_B(r_B^- m_B^-)$ *gal dcm* BL21(DE3)	Studier & Moffatt (1986)
C. glutamicum		
ATCC13032	Biotin-auxotrophic wild-type	Kinoshita *et al.* (1957)
ATCC13032Δ*chrSA*	In-frame deletion of the genes *chrS* (cg2200) and *chrA* (cg2201)	This study
ATCC13032Δ*hrtBA*	In-frame deletion of the genes *hrtB* (cg2202) and *hrtA* (cg2204)	This study
ATCC13032Δ*hrrSA*	In-frame deletion of the genes *hrrS* (cg3248) and *hrrA* (cg3247)	This study
Plasmids		
pK19*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 $oriV_{E. coli}$ *sacB lacZα)*	Schäfer *et al.* (1994)
pK19*mobsacB*-Δ*chrSA*	Kan^r, pK19*mobsacB* derivative with an overlap extension PCR product of the up- and downstream regions of *chrS* (cg2201) and *chrA* (cg2200)	This study
pK19*mobsacB*-Δ*hrtBA*	Kan^r, pK19*mobsacB* derivative with an overlap extension PCR product of the up- and downstream regions of *hrtB* (cg2202) and *hrtA* (cg2204)	This study
pJC1	Kan^r, Amp^r; *C. glutamicum* shuttle vector.	Cremer *et al.* (1990)
pJC1-*chrSA*	pJC1 derivative containing the 1.6 kbp fragment of the genes *chrA* and *chrS* and their native promoter region (250 bp)	This study
pEKEx2	Kan^r; expression vector with $lacI^q$, P_{tac} *and pUC18* multiple cloning site	Eikmanns *et al.* (1994)
pEKEx2-*hrtBA*	pEKEx2 containing the *Pst*I fragment of *hrtB and hrtA* under control of P_{tac}	This study
pET28b	Kan^r; vector for heterologous gene expression in *E. coli*, adding an N-terminal or a C-terminal hexa-histidine tag to the synthesized protein (pBR322 $oriV_{E. coli}$, P_{T7}, *lacI*)	Novagen

pET28b-*chrA*	Kan^r, pET28b-Streptag derivative containing an *Nde*I, *Hin*dIII insert of cg2200 for overproduction of ChrA (Cg2200) with an N-terminal hexa-histidine tag	This study
pJC1-P_{hrtBA}-*eyfp*	pJC1 derivative containing the promoter region (235 bp) of the genes *hrtBA* with an translational fusion to *eyfp*. The insert includes the first 24 bp of *hrtB*, a stop codon and an additional ribosome-binding site in front of *eyfp*.	This study
pJC1-P_{chrSA}-*eyfp*	pJC1 derivative containing the promoter region (235 bp) of the genes *chrSA* with an translational fusion to *eyfp*. The insert includes the first 24 bp of *chrS*, a stop codon, and an additional ribosome-binding site in front of *eyfp*.	This study
pJC1-P_{hmuO}-*eyfp*	pJC1 derivative containing the upstream region (494 bp) of the gene *hmuO* with an transcriptional fusion to *eyfp*. The insert includes the first 123 bp of *hmuO*, a stop codon, and an additional ribosome-binding site in front of *eyfp*.	This study

Table S2. Oligonucleotides used in this study

Some oligonucleotides were designed with restriction sites (underlined) as indicated or with complementary sequences for overlap PCR, shown in italics.

Oligonucleotide	**Sequence 5′–3′**	**Restriction site**
Primer for deletion plasmids		
DhrtAB-1	CGC*GGATCC*CGCCGTCAGTATTGCAATGATGA	*Bam*HI
DhrtAB-2	*CCCATCCACTAAACTTAAACA*TGCGGTGAGTTCTTTTAGTCCTTG	
DhrtAB-3	*TGTTTAAGTTTAGTGGATGGG*GGTCACCTCCTCACACCCCAC	
DhrtAB-4	ACGC*GTCGAC*CGTCTTTGGTCTGCAATGACAC	*Sal*I
DhrtBA-fw	GACCGGTGACAACGCCAACAG	
DhrtBA-rv	GATGACTGGTGGGGAACGTGAG	
DchrSA-1	TATA*GTCGAC*CACTACATCATGCGCAGTAGCG	*Sal*I
DchrSA-2	*CCCATCCACTAAACTTAAACA*AATTCGGGCGATGGTCGCTTGGC	
DchrSA-3	*TGTTTAAGTTTAGTGGATGGG*CAGCGCGGAATTATCTAGACGC	
DchrSA-4	TATA*CTGCAG*GTGCTGGTTGGCGCCCAGTTTGG	*Pst*I
DchrSA-fw	TTCATCAATACCACGGGCAGGTG	
DchrSA-rv	TTACGTTGGCTCGCTGCGCTTC	
DhrrSA-1	TATA*CCCGGG*GATGTGGCCTTCTAATAGTTAGA	*Xma*I
DhrrSA-2	*CCCATCCACTAAACTTAAACA*CAGCGAGGCTGTCAAAATGTGGA	
DhrrSA-3	*TGTTTAAGTTTAGTGGATGGG*CTCGGCGTGCGGTCACGTACC	
DhrrSA-4	TATATA*TCTAGA*TGTGTATGGTACACATTTTGTGC	*Xba*I
DhrrSA-fw	CTCCTCATGGATGTTGTGTTCCC	
DhrrSA-rv	AATCAATACACCGGCCAAGCAGG	
Oligonucleotides used for complementation		
chrSA-fw	TCTA*GCTAGC*GCGGTGAGTTCTTTTAGTC	*Nhe*I

chrSA rv	TCTAGCTAGCCTAGATAATTCCGCGCTGTC	*Nhe*I
hrtBA-RBS-fw	AAAACTGCAGAAGGAGATATAGATATGTTTCAAGGACTAAAAGAACTC	*Pst*I
hrtAB-rv	TATACTGCAGCTACAGGGTGTGGGGTGTGAG	*Pst*I
Oligonucleotides for overproduction of ChrA		
chrA-*Nde*I-fw	TATACATATGATCCGTATTCTGTTGGCTGAT	*Nde*I
chrA-*Hin*dIII-rv	TATAAAGCTTCTAGATAATTCCGCGCTGTCTGG	*Hin*dIII
Oligonucleotides for promoter fusion studies		
PchrS-*Bam*HI-fw	CGCGGATCCCGGTGAGGGCAGAGAGGAAAG	*Bam*HI
PchrS-8C-RBS-*Nde*I-rv	*CGC*CATATG*ATATCTCCTTCTTAAAGTTCA*GATGGTCGCTTGGCTAGTTTTCAC	*Nde*I
PhrtB-*Bam*HI-fw	CGCGGATCCCCACACACCCCAATGGCTGG	*Bam*HI
PhrtB-8C-RBS-*Nde*I-rv	CGCCATATG*ATATCTCCTTCTTAAAGTTCA*GAGTTCTTTTAGTCCTTGAAACAT	*Nde*I
PhmuO-*Nhe*I-fw	CTA GCTAGCGAAGTTCTTGAAGTGCTGGAAAAGC	*Nhe*I
PhmuO-RBS-rv	*CCATATATCTCCTTCTTAAAGTTCA*ATCGAGCTTCCCGGTGAGCAGATCA	
eyfp-RBS-fw	*TGAACTTTAAGAAGGAGATAT*ATGGTGAGCAAGGGCGAGGAG	
eyfp-*Nhe*I-rv	CTAGCTAGCTTATCTAGACTTGTACAGCTCG	*Nhe*I
Oligonucleotides for electrophoretic mobility shift assays		
gntK-control-fw	ATGGTGGCGTCATGCTCGGCCG	
gntK-control-rv	GGATTTGCCGCAGCCAGAAACGC	
chrS-hrtB-fw	GTGTCTGCTACTCGGTCGCGGAC	
chrS-hrtB-rv	CACCAACACCAACAAAACGGC	
hmuO-fw	ATGCGCTTGTGCTGGTCAGGGG	
hmuO-rv	TCGGCCTCTTCATGGGCCTGCGC	
hrrA-fw	TAACCTACGAAGACACAGAAG	
hrrA-rv	AGACTTCGCCCACCACTTCAATG	
chrS-hrtB-motif-fw	TTCTTGCAGTACGACCAAAGTCGGATTCGC	
chrS-hrtB-motif-rv	GCGAATCCGACTTTGGTCGTACTGCAAGAA	
hrrA_motif_fw	AAGGCTAGACTAAAGTACGATTCATCTGCT	
hrrA_motif_rv	AGCAGATGAATCGTACTTTAGTCTAGCCTT	
hmuO-motif-fw	AATTGTTCCAACTAAGGGACTATATGTAGG	
hmuO-motif-rv	CCTACATATAGTCCCTTAGTTGGAACAATT	
CgtR8_M1_for	TTCAACGAGTACGACCAAAGTCGGATTCGC	
CgtR8_M1_rev	TTCTTGCAGTACGACCAAAGTCGGATTCGC	
CgtR8_M2_for	TTCTTGCTCATCGACCAAAGTCGGATTCGC	
CgtR8_M2_rev	GCGAATCCGACTTTGGTCGATGAGCAAGAA	
CgtR8_M3_for	TTCTTGCAGTAGCTGCAAAGTCGGATTCGC	
CgtR8_M3_rev	GCGAATCCGACTTTGCAGCTACTGCAAGAA	

CgtR8_M4_for	TTCTTGCAGTACGACGTTTGTCGGATTCGC
CgtR8_M4_rev	GCGAATCCGACAAACGTCGTACTGCAAGAA
CgtR8_M5_for	TTCTTGCAGTACGACCAAACAGCGATTCGC
CgtR8_M5_rev	GCGAATCGCTGTTTGGTCGTACTGCAAGAA
CgtR8_M6_for	TTCTTGCAGTACGACCAAAGTCGCTAACGC
CgtR8_M6_rev	GCGTTAGCGACTTTGGTCGTACTGCAAGAA
CgtR8_M7_for	TTCTTGCAGTACGACCAAAGTCGGATTGCG
CgtR8_M7_rev	CGCAATCCGACTTTGGTCGTACTGCAAGAA
CgtR8_M8_for	AAGTTGCAGTACGACCAAAGTCGGATTCGC
CgtR8_M8_rev	GCGAATCCGACTTTGGTCGTACTGCAACTT

Table S3. Promoters of putative ChrA target genes.

Gene ID	Gene*	Promoter sequence	TS position†
cg2201-cg2200	*chrS*, *chrA*	agtacgaccaaagtcgga**TTcgCg**ttcatacttagttgatc**TATcgT**ggtggc**G**	nt 2 095 028, leaderless
cg2202-cg2204	*hrtB*, *hrtA*	gaatcgaccagagcccga**TTaAaA**aatgcccccgcgcaac**gAaAcT**agtaatc**A**	nt 2 095 139, leaderless
cg2445	*hmuO*	ttgttccaactaagggactatatgtaggtgtgggtaacctaa**GtTAatcT**tttgtg**A**	nt 2 331 195
cg3247	*hrrA*	gaggttgaactaagttctccaactgacgatgagtaaggc**TAGACT**aaagtac**G**	nt 3 109 880
cg3248	*hrrS*	caagcggtgagcatgatggaagcagcgaggatagtaggTAATGTacgacgc**A**	nt 3 111 231

*Operon structures controlled by the same promoter are shown in one box. The −10 and −35 regions are highlighted in bold, conserved bases in capitals. The stop codon of cg3248 (*hrrS*) is underlined. The end of the sequence represents the first base of the start codon.

†For the position of the transcriptional start the coryneregnet annotation (www.coryneregnet.de) for *C. glutamicum* ATTC 13032 was used.

Supplementary References

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6.2 Supplementary material – Phosphatase activity ensures specificity

Phosphatase activity of the histidine kinases ensures pathway specificity of the ChrSA and HrrSA two-component systems in *Corynebacterium glutamicum*

Table S1. Plasmids used in this study.

Plasmids	Characteristics	Reference
pK19*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 *oriV*$_{E. coli}$ *sacB lacZα)*	(Schäfer *et al.*, 1994)
Plasmids used for deletion	**Kan^r, pK19*mobsacB* derivative with an overlap extension PCR product of the up- and downstream regions of:**	
pK19*mobsacB*-Δ*hrrSA*	*hrrS* (cg3248) and *hrrA* (cg3247)	(Heyer *et al.*, 2012)
pK19*mobsacB*-Δ*chrSA*	*chrS* (cg2201) and *chrA* (cg2200)	(Heyer *et al.*, 2012)
pK19*mobsacB*-Δ*hrrS*	*hrrS* (cg3248)	This work
pK19*mobsacB*-Δ*hrrA*	*hrrA* (cg3247)	(Frunzke *et al.*, 2011)
pK19*mobsacB*-Δ*chrS*	*chrS* (cg2201)	This work
pK19*mobsacB*-Δ*chrA*	*chrA* (cg2200)	This work
Plasmids used for amino acid exchanges		
pK19*mobsacB-hrrA*-D54A	Kan^r, pK19*mobsacB* derivative with a fragment covering the first 368 bp of *hrrA* (cg3247) and 52 bp of the upstream region, including the point mutation for the amino acid exchange (aspartate to alanine) in *hrrA* at position 54	This work
pK19*mobsacB-chrA*-D54A	Kan^r, pK19*mobsacB* derivative with a fragment covering the first 370 bp of *chrA* (cg2200) and 57 bp of the upstream region, including the point mutation for the amino acid exchange (aspartate to alanine) in *chrA* at position 54	This work
pK19*mobsacB-hrrS*-Q222A	Kan^r, pK19*mobsacB* derivative with a fragment covering 426 bp of *hrrS* (cg3248), including the point mutation for the amino acid exchange (glutamine to alanine) in *hrrS* at position 222	This work
pK19*mobsacB-chrS*-Q191A	Kan^r, pK19*mobsacB* derivative with a fragment covering 427 bp of *chrS* (cg2201), including the point mutation for the amino acid exchange (glutamine to alanine) in *chrS* at position 191	This work
Plasmids used for complementation		
pJC1	Kan^r, Amp^r; *C. glutamicum* shuttle vector.	(Cremer *et al.*, 1990)
pJC1-*chrSA*	pJC1 derivative containing the 1.6 kbp fragment	(Heyer *et al.*, 2012)

	of the genes *chrA* and *chrS* (cg2200 and cg2201) including their native promoter region	
pJC1-*hrrSA*	pJC1 derivative containing the 2.191 kbp fragment of the genes *hrrA* and *hrrS* (cg3248 and cg3247) including their native promoter regions	This work
pEKEx2 (px2)	Kan^r; expression vector with *lacI*^{q}, P_{tac} *and pUC18* multiple cloning site	(Eikmanns *et al.*, 1994)
px2-*hrtBA*	px2 containing *hrtB and hrtA* (cg2202 and cg2204) under control of P_{tac}	(Heyer *et al.*, 2012)
px2-*hmuO*	px2 containing *hmuO* (cg2445) under control of P_{tac}	This work
Plasmids used for overproduction		
pET28b	Kan^r; vector for heterologous gene expression in *E. coli*, adding an N-terminal or a C-terminal hexahistidine tag to the synthesized protein (pBR322 *ori*$V_{E.coli}$, P_{T7}, *lacI*)	Novagen
pET28b-*chrA*	Kan^r, pET28b-Streptag derivative containing an insert of *chrA* (cg2200) for over-production of ChrA with an N-terminal hexahistidine tag.	(Heyer *et al.*, 2012)
pET28b-*hrrA*	Kan^r pET28b-Streptag derivative containing an insert of *hrrA* (cg3247) for overproduction of HrrA with an N-terminal hexahistidine tag	(Frunzke *et al.*, 2011)
pET16b	Amp^r;P_{T7} *lacI oriV* from pBR322; *E. coli* expression vector for overproduction of proteins with an N-terminal decahistidine tag that can be cleaved off by factor Xa	Novagen
pET16b-*phoR*	Amp^r; pET16b derivative containing an insert of *phoR* (cg2888) for overproduction of PhoR with an N-terminal decahistidinetag	(Schaaf and Bott, 2007)
pMal-c	Amp^r;P_{tac} *lacI*^{q} ColE1 *oriV*; *E. coli* expression vector for construction and overproduction of fusion proteins containing the MBP of *E. coli* (MalE) without its signal peptide	New England Biolabs
pMBP-PhoSΔ1-246	Amp^r; pMal-c derivative containing an insert of *phoS* (cg2887) kinase domain (residues 247 to 485) for overproduction of PhoS kinase domain fused to the C-terminus of the *E. coli* MBP	(Schaaf and Bott, 2007)
pMBP-HrrSΔ1-169	Amp^r; pMal-c derivative containing an insert of *hrrS* (cg3248) kinase domain (residues 170 to 444) for overproduction of the HrrS kinase domain fused to the C terminus of *E. coli* MBP	(Frunzke *et al.*, 2011)
pMBP-HrrSQ222AΔ1-169	Amp^r; pMal-c derivative containing an insert of *hrrS*-Q222A (cg3248) kinase domain (residues 170 to 444) for overproduction of the HrrSQ222A kinase domain fused to the C terminus of *E. coli* MBP	This work
pMBP-ChrSΔ1-176	Amp^r; pMal-c derivative containing an insert of *chrS* (cg2201) kinase domain (residues 177 to	This work

	377) for overproduction of the ChrS kinase domain fused to the C terminus of *E. coli* MBP	
pMBP-ChrSQ191AΔ1-176	Amp^r; pMal-c derivative containing an insert of *chrS*-Q191A (cg2201) kinase domain (residues 177 to 377) for overproduction of the ChrSQ191A kinase domain fused to the C terminus of *E. coli* MBP	This work
Plasmids used for reporter studies		
pJC1-P_{hrtBA}-*eyfp*	pJC1 derivative containing the promoter region (235 bp) of the genes *hrtBA* with an translational fusion to *eyfp*. The insert includes the first 24bp of *hrtB*, a stop codon and an additional ribosome binding site in front of *eyfp*.	(Heyer *et al.*, 2012)
pJC1-P_{hmuO}-*eyfp*	pJC1 derivative containing the upstream region (494 bp) of the gene *hmuO* with an transcriptional fusion to *eyfp*. The insert includes the first 123 bp of *hmuO*, a stop codon, and an additional ribosome binding site in front of *eyfp*.	(Heyer *et al.*, 2012)

Table S2. Oligonucleotides used in this study[a].

Oligonucleotide	Sequence 5' -> 3'	Restriction site
Oligonucleotides used for construction of deletion plasmids		
DhrrS-1	TATACCCGGGGATGTGGCCTTCTAATAGTTAGA	XmaI
DhrrS-2	*CCCATCCACTAAACTTAAACA*CAGCGAGGCTGTCAAAATGT GGA	
DhrrS-3	*TGTTTAAGTTTAGTGGATGGG*GGCGAGGTTGAACTAAGTTC TCC	
DhrrS-4	TATATCTAGAAGTGTGGAGTCACCTTCTGCTG	XbaI
DhrrS-fw	CTCCTCATGGATGTTGTGTTCCC	
DhrrS-rv	AATCAATACACCGGCCAAGCAGG	
DchrS-1	TATAGTCGACCACTACATCATGCGCAGTAGCG	SalI
DchrS-2	*CCCATCCACTAAACTTAAACA*AATTCGGGCGATGGTCGCTT GGC	
DchrS-3	*TGTTTAAGTTTAGTGGATGGG*GCCCGTTTTCCCTATCCACAA AAG	
DchrS-4	TATACTGCAGAGGACAGCACTTCAATTTCTCTGG	PstI
DchrS-fw	TTCATCAATACCACGGGCAGGTG	
DchrS-rv	TTACGTTGGCTCGCTGCGCTTC	
DchrA-1	TATACTGCAGGCGTTAAGCCATATACGGTTGG	PstI
DchrA-2	*CCCATCCACTAAACTTAAACA*CAACAGAATACGGATCACTTA TCTT	
DchrA-3	*TGTTTAAGTTTAGTGGATGGG*CAGCGCGGAATTATCTAGAC GC	
DchrA-4	TATACTGCAGGTGCTGGTTGGCGCCCAGTTTGG	PstI
DchrA-fw	TTCATCAATACCACGGGCAGGTG	
DchrA-rv	TTACGTTGGCTCGCTGCGCTTC	
Oligonucleotides used for amino acid exchanges		
hrrA-D54A-1	ATATATGAATTCCGCATTTTCATTCGAAACGAAG	EcoRI
hrrA-D54A-2	*GGCCGAATCGGAG**GGC**CATCAAGATGAC*	
hrrA-D54A-3	*GTCATCTTGATG**GCC**CTCCGATTCGGCC*	
hrrA-D54A-4	ATATATGGATCCACTGCTGCCAGGAGTTCGCTC	BamHI
chrA-D54A-1	ATATATGAATTCCGGATCGGGAACTGGAATTTC	EcoRI
chrA-D54A-2	*CAAAACGCAG**AGCC**ATCAACACCACATC*	
chrA-D54A-3	*GATGTGGTGTTGATG**GCT**CTGCGTTTTG*	
chrA-D54A-4	TATATAGGATCCCATCGCGAACACCGGCAATGAG	BamHI
hrrS-Q222A-1	CGCGGATCCCCTTTGGTGGTGTGATGGGTC	BamHI
hrrS-Q222A-2	*GACACGGTCGCC**GCG**GGACTCTCCTCCATTC*	
hrrS-Q222A-3	*GAATGGAGGAGAGTCC**CGC**GGCGACCGTGTC*	

hrrS-Q222A-4	CGCGAATTCCGCGGTGAAGTGCTGCTTCC	EcoRI
hrrS-fw	CGATTGCCAGCCAGTATTCC	
hrrS-rv	GCAGTTGGCGAACATCACCG	
chrS-Q191A-1	CGCGGATCCCGTTGGCTATGCACCTGGGG	BamHI
chrS-Q191A-2	*GACACTGTGGCG**GCG**GGGTTGTCCTCG*	
chrS-Q191A-3	*CGAGGACAACCC**CGC**CGCCACAGTGTC*	
chrS-Q191A-4	CGCGAATTCCCCATCGGGGTGGTCGACG	EcoRI
chrS-fw	GCCTGCGTATTTGGTGTTTC	
chrS-rv	CTGTGCATGCCGCACCACAT	
Oligonucleotides used for construction of complementation plasmids		
hrrSA-fw	CTAGCTAGCCTACAGCAGCCCCTGCTCAC	NheI
hrrSA-rv	CTAGCTAGCCGTCAGTAATTACTTCCGGGG	NheI
hmuO-RBS-fw	ATATATGGATCCAAGGAGATATAGATATGACAAGCATTATTG CAAGC	BamHI
hmuO-rv	TATATAGAATTCTTAAGCAAGAGCCTGAAAAAC	EcoRI
Oligonucleotides used for overproduction of ChrS		
chrS-K-fw	ATATATCTGCAGTTTCAGTTATTGGCACAAGCC	PstI
chrS-K-rv	tatataaagcttTCACTTATCTTGGTCCTTTTGTG	HindIII

[a] Some oligonucleotides were designed with restriction sites (underlined) as indicated or with complementary sequences for overlap PCR, shown in italics, mutations for amino acid exchanges shown in bold.

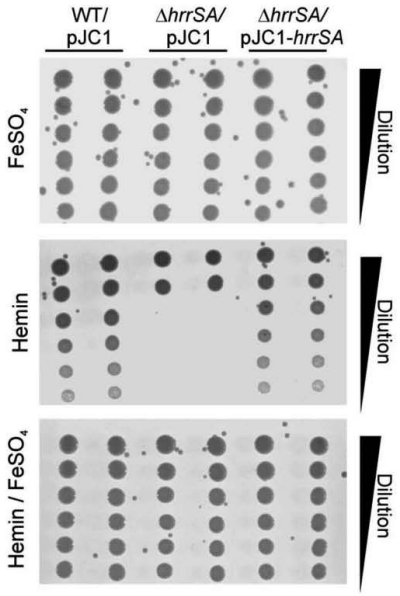

Fig. S1. Growth phenotypes of *C. glutamicum* ATCC 13032 (WT) and Δ*hrrSA* mutants. The strains harbored the indicated plasmids (pJC1 or pJC1-*hrrSA*). For growth on agar plates cells were diluted in 0.9% (w/v) NaCl to an OD_{600} of 1 and dilution series (3 µl each, 10^0 to 10^{-5}) were spotted on CGXII agar plates containing either 2.5 µM $FeSO_4$ (+PCA, iron available), 2.5 µM hemin (-PCA, strong iron limitation), or 2.5 µM $FeSO_4$ and 2.5 µM hemin (+PCA, iron available). Plates were incubated at 30 °C for 48 h and photographed.

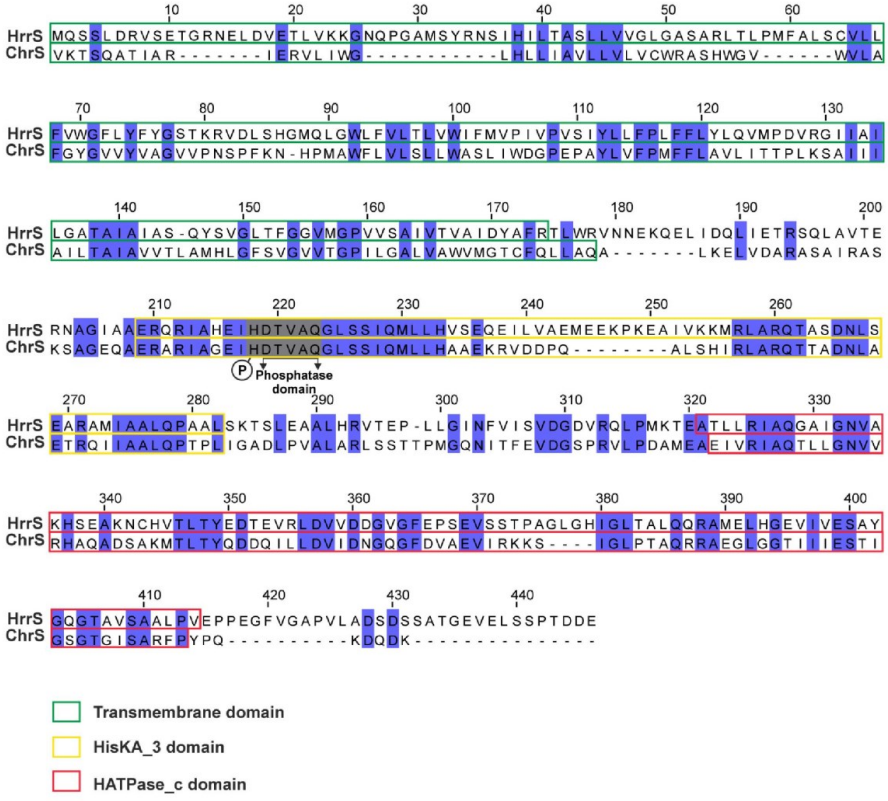

Figure S2. Alignment and domain architecture of the kinases HrrS and ChrS from *C. glutamicum*. The alignment was performed using the online tool ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/). Identical amino acids are marked in blue, conserved histidine residue and the phosphatase domain are shaded in grey. HisKA_3 (yellow) and HATPase_c domains (red) were obtained from the Pfam database (http://pfam.sanger.ac.uk/). Transmembrane regions were predicted using TopPredII (http://bioweb.pasteur.fr/seganal/interfaces/toppred.html) and HMMTOP (http://www.sacs.ucsf.edu/cgi-bin/hmmtop.py).

6.3 Supplementary material – Identification of phosphatase residues

Identification of residues involved in phosphatase activity of the two-component systems HrrSA and ChrSA in *Corynebacterium glutamicum*

Table S1. Plasmids used in this study.

Plasmids	Characteristics	Reference
pK19*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 *oriV$_{E. coli}$ sacB lacZα)*	(Schäfer *et al*., 1994)
pK18*mobsacB*	Kan^r; vector for allelic exchange in *C. glutamicum* (pK18 *oriV$_{E. coli}$ sacB lacZα)*	(Schäfer *et al*., 1994)
pJC1	Kan^r; Amp^r *C. glutamicum* shuttle vector	(Cremer *et al*., 1990)
Plasmids used for deletion		
pK19*mobsacB*-Δ*hrrS*	Kan^r, pK19mobsacB derivative with an overlap extension PCR product of the up- and downstream regions of *hrrS* (cg3248)	(Hentschel *et al*., 2014)
pK19*mobsacB*-Δ*chrS*	Kan^r, pK19mobsacB derivative with an overlap extension PCR product of the up- and downstream regions of *chrS* (cg2201)	(Hentschel *et al*., 2014)
Plasmids used for aminoacid exchanges		
pK19*mobsacB-hrrS*-Q222A	Kan^r, pK19*mobsacB* derivative with a fragment covering 426 bp of *hrrS* (cg3248), including the point mutation for the amino acid exchange (glutamine to alanine) in *hrrS* at position 222	(Hentschel *et al*., 2014)
pK19*mobsacB-chrS*-Q191A	Kan^r, pK19*mobsacB* derivative with a fragment covering 427 bp of *chrS* (cg2201), including the point mutation for the amino acid exchange (glutamine to alanine) in *chrS* at position 191	(Hentschel *et al*., 2014)
Plasmids used for chimera construction in phosphatase OFF strains		
pK19mobsacB-*hrrS*Ex1	pK19 derivative containing a *hrrS* fragment encoding for the HrrS DHp domain (HrrS 208-281) and the corresponding up- and downstream flanking regions of *chrS* (encoding for ChrS 96-176 and 243-323) for the exchange of the ChrS DHp domain against the HrrS DHp domain.	This work
pK19mobsacB-*hrrS*Ex2	pK19 derivative containing a *hrrS* fragment encoding for a part of the HrrS DHp domain (HrrS 233-281) and the corresponding up- and downstream flanking regions of *chrS* (encoding	This work

	for ChrS 121-201 and 243-323) for the exchange of the ChrS DHp domain against the HrrS DHp domain.	
pK19mobsacB-*hrrS*Ex3	pK19 derivative containing a *hrrS* fragment encoding for a part of the HrrS DHp domain (HrrS 233-255) and the corresponding up- and downstream flanking regions of *chrS* (encoding for ChrS 121-201 and 217-247) for the exchange of the ChrS DHp domain against the HrrS DHp domain.	This work
pK19mobsacB-*chrS*Ex1	pK19 derivative containing a *chrS* fragment encoding for the ChrS DHp domain (ChrS 177-242) and the corresponding up- and downstream flanking regions of *hrrS* (encoding for HrrS 127-207 and 282-362) for the exchange of the HrrS DHp domain against the ChrS DHp domain.	This work
pK19mobsacB-*chrS*Ex2	pK19 derivative containing a *chrS* fragment encoding for a part of the ChrS DHp domain (ChrS 202-242) and the corresponding up- and downstream flanking regions of *hrrS* (encoding for HrrS 152-232 and 282-362) for the exchange of the HrrS DHp domain against the ChrS DHp domain.	This work
pK19mobsacB-*chrS*Ex3	pK19 derivative containing a *chrS* fragment encoding for a part of the ChrS DHp domain (ChrS 202-216) and the corresponding up- and downstream flanking regions of *hrrS* (encoding for HrrS 152-232 and 256-336) for the exchange of the HrrS DHp domain against the ChrS DHp domain.	This work
Plasmids used for reporter integration in intergenic region between cg1121/22		
pK18-P_{hmuO}-*venus*	pK18 derivative containing the upstream region (494 bp) of the gene *hmuO* with an transcriptional fusion to *venus.* The insert includes the first 123 bp of *hmuO*, a stop codon, and an additional ribosome binding site in front of *venus.*	This work
Plasmids used for library construction		
pJC1-*hrrS*	pJC1 derivative with a fragment covering *hrrS* (cg3248), including the promoter region of *hrrS* (200 bp upstream).	This work
pJC1-*hrrS*-DHp	pJC1 derivative with a fragment covering *hrrS* (cg3248) including the promoter region of *hrrS* (200 bp upstream) with the error proned DHp domain of *hrrS* (208-281).	This work
pJC1-*hrrS*-DHp-left	pJC1 derivative with a fragment covering *hrrS* (cg3248) including the promoter region of *hrrS* (200 bp upstream) with the left part of the error proned DHp domain of *hrrS* (208-232)	This work

Plasmids used for reporter studies		
pJC1-P_{hrtBA}-*eyfp*	pJC1 derivative containing the promoter region (235 bp) of the genes *hrtBA* with an translational fusion to *eyfp.* The insert includes the first 24bp of *hrtB*, a stop codon and an additional ribosome binding site in front of *eyfp*.	(Heyer *et al*.,2012)
pJC1-P_{hmuO}-*eyfp*	pJC1 derivative containing the upstream region (494 bp) of the gene *hmuO* with an transcriptional fusion to *eyfp.* The insert includes the first 123 bp of *hmuO*, a stop codon, and an additional ribosome binding site in front of *eyfp.*	(Heyer *et al*.,2012)

Table S1. Oligonucleotides used in this study[a]

Oligonucleotide	**Sequence**
HrrSep-DHp-fw	*GAACGAAATGCGGGTATTGCTG*
HrrSep-DHp-rv	*GCTGCTTCCAAGGAGGTTTTAGA*
HrrS-DHp-1-fw	*GATCAGCGACGCCGCAGGGG*CGGCGACCCAGTCGGTG
HrrS-DHp-2-rv	*CGCAGCAATACCCGCATTTCGTTC*
HrrS-DHp-3-fw	TCTAAAACCTCCTTGGAAGCAGC
HrrS-DHp-4-rv	TTGCCATTGCTGCAGGTCGATTACTCA*TCGTCAGTTGGAGAACTTAG*
HrrSep-DHpleft-fw	*GAACGAAATGCGGGTATTGCTG*
HrrSep-DHpleft-rv	*CAACGAGAATCTCCTGTTCAGAGAC*
HrrS-DHp-left-1-fw	*GATCAGCGACGCCGCAGGGG*CGGCGACCCAGTCGGTG
HrrS-DHp-left-2-rv	*CGCAGCAATACCCGCATTTCGTTC*
HrrS-DHp-left-3-fw	*GTCTCTGAACAGGAGATTCTCGTTG*
HrrS-DHp-left-4-rv	TTGCCATTGCTGCAGGTCGATTACTCA*TCGTCAGTTGGAGAACTTAG*
HrrS-fw	*GATCAGCGACGCCGCAGGGG*CGGCGACCCAGTCGGTG
HrrS-rv	TTGCCATTGCTGCAGGTCGATTACTCA*TCGTCAGTTGGAGAACTTAG*
PhmuO-fw	*GAATTCCTCTTGCTCGTGTC*GAAGTTCTTGAAGTGCTGG
PhmuO-RBS-rv	*ATATCTCCTTCTTAAAGTTCA*ATCGAGCTTCCCGGTGAGCAG
Venus-RBS-fw	*TGAACTTTAAGAAGGAGATAT*ATGGTGAGCAAGGGCG
Venus-rv	*AATTGTGTCCATGAGTTCGC*TCGATTACTTGTACAGCTCGTCCATG
Oligonucleotides for construction of HrrSEx1	
HrrS-Ex1-fw	*AAAGCGCTGGCGAGCAGGCAGAACGTCAACGTATTGCG*
HrrS-Ex1-rv	*ACCGGCAGATCCGCCCCAATCAGCGCTGCCGGTTG*

ChrS-1-fw	*CCTGCAGGTCGACTCTAGAG*GTGTTGATCACGACACCG
ChrS-2-rv	*TGCGCAATACGTTGACGTTCTGCCTGCTCGCCAGC*
ChrS-3-fw	*CGTTGCAACCGGCAGCGCTGATTGGGGCGGATCTGC*
ChrS-4-rv	*TTGTAAAACGACGGCCAGTGAATT*CCCATTATCGATGACATCTAG
Oligonucleotides for construction of HrrSEx2	
HrrS-Ex2-fw	*CGATTCAGATGTTGTTGCATGTCTCTGAACAGGAGATTCTCG*
HrrS-Ex2-rv	*ACCGGCAGATCCGCCCCAATCAGCGCTGCCGGTTG*
ChrS-1-fw	*CCTGCAGGTCGACTCTAGAG*ATGCACCTGGGGTTTTC
ChrS-2-rv	*AGAATCTCCTGTTCAGAGACATGCAACAACATCTGAATCGAG*
ChrS-3-fw	*CGTTGCAACCGGCAGCGCTGATTGGGGCGGATCTGC*
ChrS-4-rv	*TTGTAAAACGACGGCCAGTGAATT*CCCATTATCGATGACATCTAG
Oligonucleotides for construction of HrrSEx3	
HrrS-Ex3-fw	*CGATTCAGATGTTGTTGCATGTCTCTGAACAGGAGATTCTCG*
HrrS-Ex3-rv	*GTCGTTTGCCTGGCCAACCGCATCTTCTTCACGATCGCCTC*
ChrS-1-fw	*CCTGCAGGTCGACTCTAGAG*ATGCACCTGGGGTTTTC
ChrS-2-rv	*AGAATCTCCTGTTCAGAGACATGCAACAACATCTGAATCGAG*
ChrS-3-fw	AGGCGATCGTGAAGAAGATGCGGTTGGCCAGGCAAAC
ChrS-4-rv	*TTGTAAAACGACGGCCAGTGAATT*CCTGTGCATGCCGCAC
Oligonucleotides for construction of ChrSEx1	
ChrS-Ex1-fw	*GAAATGCGGGTATTGCTGCGGAACGAGCCCGCATAGC*
ChrS-Ex1-rv	*GCTTCCAAGGAGGTTTTAGAGAGTGGAGTCGGTTGCAG*
HrrS-1-fw	*CCTGCAGGTCGACTCTAGA*GGTGAGAGGCATTATTGCGATTTTG
HrrS-2-rv	*CCCGCTATGCGGGCTCGTTCCGCAGCAATACCCGCATTTC*
HrrS-3-fw	*CGCTGCAACCGACTCCACTCTCTAAAACCTCCTTGGAAGC*
HrrS-4-rv	*TTGTAAAACGACGGCCAGTGAATT*CACACCGTCATCAACCAC
Oligonucleotides for construction of ChrSEx2	
ChrS-Ex2-fw	*CCATTCAAATGCTGCTGCATGCGGCGGAAAAACGG*
ChrS-Ex2-rv	*GCTTCCAAGGAGGTTTTAGAGAGTGGAGTCGGTTGCAG*
HrrS-1-fw	*CCTGCAGGTCGACTCTAGAG*GGTGGTGTGATGGGTCC
HrrS-2-rv	*TCCACCCGTTTTTCCGCCGCATGCAGCAGCATTTGAATGG*
HrrS-3-fw	*CGCTGCAACCGACTCCACTCTCTAAAACCTCCTTGGAAGC*
HrrS-4-rv	*TTGTAAAACGACGGCCAGTGAATT*CACACCGTCATCAACCAC
Oligonucleotides for construction of ChrSEx3	
ChrS-Ex3-fw	*CCATTCAAATGCTGCTGCATGCGGCGGAAAAACGG*

ChrS-Ex3-rv	*GCTGTTTGTCGGGCAAGGCGTATATGGCTTAACGCCTGC*
HrrS-1-fw	*CCTGCAGGTCGACTCTAGAG*GGTGGTGTGATGGGTCC
HrrS-2-rv	*TCCACCCGTTTTTCCGCCGCATGCAGCAGCATTTGAATGG*
HrrS-3-fw	*CGCAGGCGTTAAGCCATATACGCCTTGCCCGACAAAC*
HrrS-4-rv	*TTGTAAAACGACGGCCAGTGAATT*ATGTTTCGCCACATTTCCG

[a] Some oligonucleotides were designed to introduce overlaps with complementary sequences for Gibson cloning, shown in italics.

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Hentschel, E., Mack, C., Gätgens, C., Bott, M., Brocker, M. and Frunzke, J., (2014) Phosphatase activity of the histidine kinases ensures pathway specificity of the ChrSA and HrrSA two-component systems in *Corynebacterium glutamicum*. *Mol Microbiol* **92**: 1326-1342.

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6.4 Supplementary material – Signal perception of HrrS and ChrS

Figure S1. Reporter studies of the haem importer deletion mutant Δ*hmuTUV*/Δ*htaA*

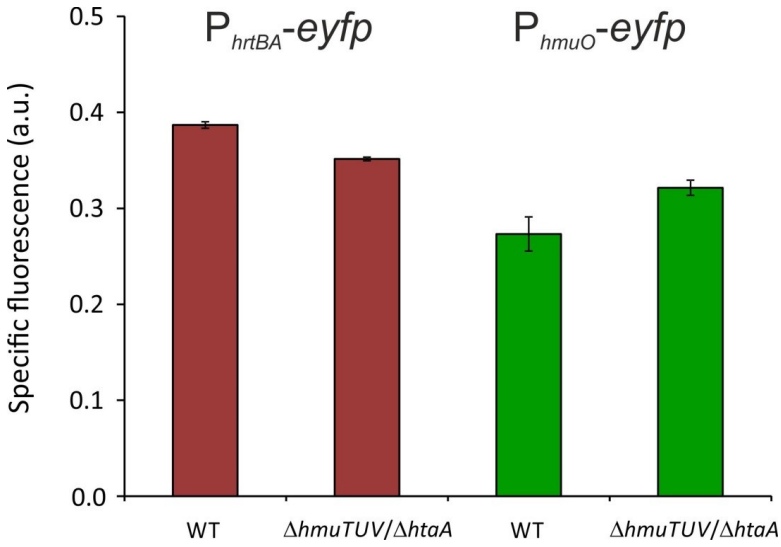

Fig. S1: Deletion of *hmuTUV*/*htaA* leads to no alteration of target gene reporter activity of P_{hrtBA}-*eyfp* and P_{hmuO}-*eyfp* *in C. glutamicum* ATCC13032. Strains carrying the reporter plasmids were inoculated to an OD_{600} of 1 in 750 µl of CGXII minimal medium containing 4% glucose and cultivated in the BioLector system in CGXII minimal medium with 2.5 µM hemin. Fluorescence was recorded after 2.5 (P_{hrtBA}-*eyfp*) and 8 h (P_{hmuO}-*eyfp*) respectively. In the BioLector system the growth (backscatter signal of 620 nm light) and eYFP fluorescence (excitation 510 nm/emission 532 nm) were monitored in 10 min intervals. The specific fluorescence was calculated as fluorescence signal per backscatter signal (given in arbitrary units, a.u.). Results represent average values with standard deviation of three independent experiments.

6.5 Supplementary material – Interaction of HrrSA and ChrSA

Figure S2. Bacterial two hybrid assay of the TCS HrrSA and ChrSA

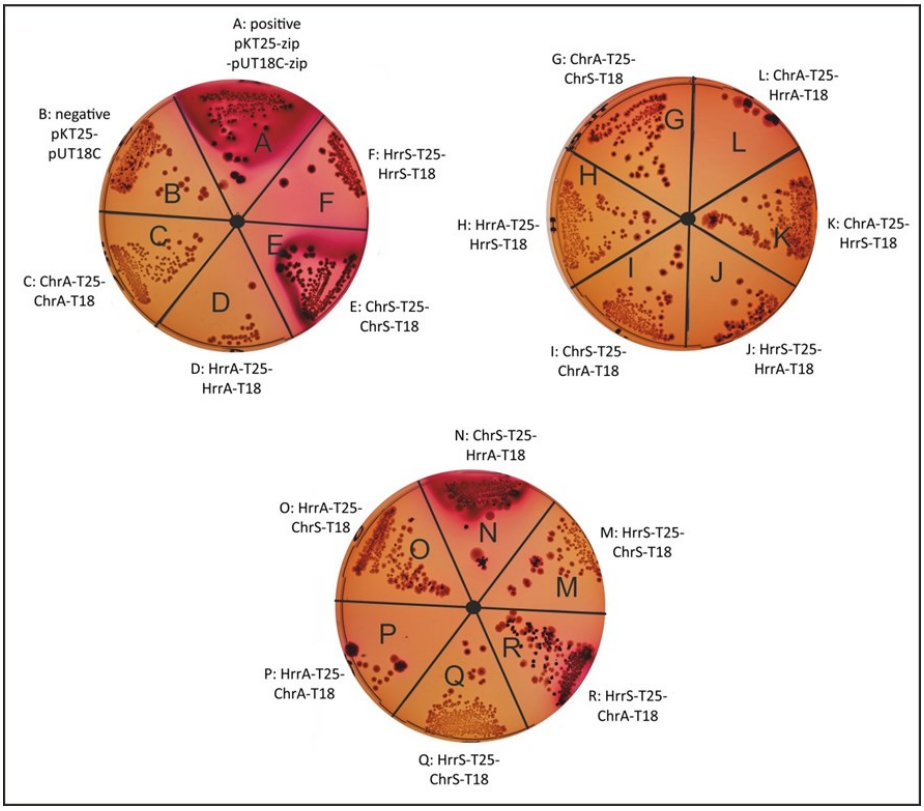

Fig. S2: Heterologous protein-protein interaction studies of the TCS HrrSA and ChrS from *C. glutamicum* in *E. coli*. Bacterial two-hybrid assay was performed according to the suppliers manual (Euromedex, Souffelweyersheim, France). HrrS, ChrS, HrrA, and ChrA were fused to the T25 fragment and to the T18 fragment of the adenylate cyclase. *E. coli* DHM1 strains were co-transformed with the plasmids pKT25 and pUT18C containing the genes encoding for the protein fusions as indicated. Cells were plated on McConkey/maltose plates containing ampicillin and kanamycin and were incubated for 24-72h at 30 °C. A stron interaction could be observed for HrrS-HrrS and ChrS-ChrS in (F and E) indicating that they form homodimers. Furthermore, a strong interaction could be observed for ChrS-HrrA and HrrS-ChrA (N and R) confirms a potential cross-talk between HrrSA and ChrSA.

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